2016

Discriminative Stimulus Properties of Endogenous Cannabinoid Degradative Enzyme Inhibitors

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Discriminative Stimulus Properties of Endogenous Cannabinoid Degradative Enzyme Inhibitors

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

By

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April 2016

Acknowledgements

The author wishes to thank several people. I would like to thank my dissertation advisor Dr. Aron Lichtman for allowing me to join his lab as a post-baccalaureate student in 2010, and sticking with me as a doctoral student. There have been many ups and downs during my graduate training, and he has always been a staunch supporter of me and of my career aspirations. In addition to his positive influence on my scientific training, Dr. Lichtman has been an excellent mentor and has become a second father figure. Words cannot express how thankful I am, and a large portion of my future career accomplishments will be because of him. Additionally, I would like to thank all of my committee members (Drs. Francine Cabral, Patrick Beardsley, Imad Damaj, Joe Porter, Suzanne Barbour) for your willingness to participate on my committee. Dr. Beardsley and Dr. Porter, thank you for our one on one conversations to help me understand some of the more debatable concepts in drug discrimination, and for your feedback on my manuscript. Dr. Cabral, you have always brought positive energy to our meetings, and our personal interactions when we crossed paths in the cafeteria, when I needed it the most.

Thank you to my big four. In the sport of professional basketball, there has been a common idea over the past several years, that a team with a nucleus of three superstars will have a very good chance of winning a championship. Instead of a big three, I have a big four (Drs. Lichtman, Lloyd, Barbour, Wu-Pong) that have been crucial to getting me to my dissertation defense (my personal championship game) on July 15th and preparing me for the next chapter in my life. Dr. Lloyd has done a tremendous job running the VCU post-baccalaureate research

program. The PREP program gave me the opportunity to spend one year in Dr. Lichtman's lab after undergrad to learn how to conduct graduate level research, and determine if I wanted to pursue a Ph.D. Dr. Barbour was someone who I could confide in, share my struggles, and provided an enormous amount of support and guidance. Without her presence, I'm not sure if I would have made it past the first year of graduate school. Dr. Wu-Pong opened my eyes to a larger world, and established a program to help students that will not have careers in academia, but instead choose non-traditional career paths to discover their best self, and have more confidence in their career goals. I believe most people will fall into non-traditional paths by serendipity, and Dr. Wu-Pong has made this process more natural.

Last but certainly not least, I want to thank my parents. My mom is one of the hardest working women I have met in my entire life. From the family farm picking cotton, vegetables and raising livestock, to quality control manager at Asahi Kasei, her work ethic has always been second to none. After being diagnosed with breast cancer, she drove from work during her lunch breaks to receive chemotherapy and immediately went back to work, never using her situation as an excuse to miss work. My father was born in the Deep South during the time period of Jim Crow and was able to escape poverty and obtain a very respectable career in banking. Above all, my parents have inspired me to never quit.

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2-AG	2-arachidonoylglycerol
AA	arachidonic acid
ABHD	α/β hydrolase
AC	adenylyl cyclase
AEA	anandamide
ANOVA	analysis of variance
Bmax	Maximal specific binding sites
cAMP	Cyclic adenosine monophosphate
CB_1	Cannabinoid receptor, subtype 1
CB_2	Cannabinoid receptor, subtype 2
DAGL	Diacylglycerol lipase
eCB	endocannabinoid
FAAH	Fatty acid amide hydrolase
G-protein	Guanine nucleotide binding protein
Gi	cAMP inhibitory G-protein

GPCR	G-protein coupled receptor
i.p.	Intraperitoneal
JZL184	4-nitrophenyl-4-(dibenzo[d][1,3]dioxol-5-yl(hydroxy)methyl) piperidine-1-carboxylate
JZL195	4-nitrophenyl 4-(3-phenoxybenzyl) piperazine-1-carboxylate
KT195	([4-(4′-Methoxy[1,1′-biphenyl]-4-yl)-1H-1,2,3-triazol-1-yl](2-phenyl-1-piperidinyl)-methanone)
KT182	([4-[3′-(Hydroxymethyl)[1,1′-biphenyl]-4-yl]-1H-1,2,3-triazol-1-yl](2-phenyl-1-piperidinyl)-methanone)
MAGL	Monoacylglycerol lipase
MJN110	(2,5-dioxopyrrolidin-1-yl 4-(bis(4-chlorophenyl)methyl)piperazine-1-carboxylate)
NAPE-PLD	N-acyl phosphatidylethanolamine phospholipase D
OEA	Oleylethanolamine
PEA	Palmitoylethanolamine
PF-3845	N-(pyridin-3-yl)-4-(3-(5-(trifluoromethyl)pyridin-2-yloxy)benzyl) piperdine-1-carboxamide

Rim	Rimonabant (SR141716A)
SA-57	(4-[2-(4-Chlorophenyl)ethyl]-1-piperidinecarboxylic acid 2-(methylamino)-2-oxoethyl ester)
SAMHSA	Substance Abuse and Mental Health Services Administration
s.c.	Subcutaneous
SR144528	(N-[(1S)-endo-1,3,3-trimethylbicyclo [2.2.1]heptan2-yl]-5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-1H-pyrazole-3-carboxamide)
THC	Δ_9-tetrahydrocannabinol
URB597	[3-(3-carbamoylphenyl)phenyl] N-cyclohexylcarbamate
WIN 55, 212	(R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo[1,2,3-de)-1,4-benzoxazin-6-yl]-1-napthalenylmethanone

Abstract

DISCRIMINATIVE STIMULUS PROPERTIES OF ENDOGENOUS CANNABINOID DEGRADATIVE ENZYME INHIBITORS

By Allen Owens, Ph.D.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2016

Major Director: Dr. Aron Lichtman, Professor, Department of Pharmacology & Toxicology; Associate Dean for Research and Graduate Studies, School of Pharmacy

Inhibition of fatty acid amide hydrolase (FAAH) or monoacylglycerol lipase (MAGL), the chief degradative enzymes of N-arachidonoyl ethanolamine (anandamide; AEA) and 2-arachidonoylglycerol (2-AG), respectively, elicits no or partial substitution for Δ^9-tetrahydrocannabinol (THC) in drug discrimination procedures. However, combined inhibition of both enzymes fully substitutes for THC, as well as produces a full constellation of cannabimimetic effects. Because no published report to date have investigated whether an inhibitor of endocannabinoid hydrolysis will serve as a discriminative stimulus, the purpose of this doctoral dissertation was to investigate whether C57BL/6J mice would learn to discriminate SA-57 (4-[2-(4-Chlorophenyl)ethyl]-1-piperidinecarboxylic acid 2-(methylamino)-2-oxoethyl

ester), a dual inhibitor of FAAH and MAGL, from vehicle in the drug discrimination paradigm. Also, we sought to determine whether inhibiting both enzymes, or inhibiting one enzyme was necessary to generate the SA-57 discriminative stimulus. Initial experiments showed that SA-57 fully substituted for either CP 55,940 ((-)-cis-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol), a high efficacy CB_1 receptor agonist in C57BL/6J, mice or AEA in FAAH $^{(-/-)}$ mice. The majority (i.e., 23 of 24) of subjects achieved criteria of discriminating SA-57 (10 mg/kg) from vehicle within 40 sessions, with full generalization occurring 1-2 h post injection. CP 55,940, the dual FAAH-MAGL inhibitor JZL195 (4-nitrophenyl 4-(3-phenoxybenzyl)piperazine-1-carboxylate), the MAGL inhibitors MJN110 (2,5-dioxopyrrolidin-1-yl 4-(bis(4-chlorophenyl)methyl)piperazine-1-carboxylate) and JZL184 (4-[*Bis*(1,3-benzodioxol-5-yl)hydroxymethyl]-1-piperidinecarboxylic acid 4-nitrophenyl ester) fully substituted for SA-57. Although, the FAAH inhibitors PF-3845 and URB597 did not substitute for SA-57, PF3845 produced a two-fold leftward shift in the MJN110 substitution dose-response curve. In addition, the CB_1 receptor antagonist rimonabant blocked the generalization of SA-57 as well as substitution of CP 55,940, JZL195, MJN110, JZL184 for the SA-57 discriminative stimulus. These findings taken together indicate that the inhibition of endocannabinoid-regulating enzymes serve as breaks to prevent overstimulation of CB_1 receptors, and MAGL inhibition is the major driving force for generating the SA-57 discriminative stimulus.

Chapter 1. Introduction

Cannabis sativa (marijuana) has been cultivated for thousands of years for its therapeutic benefits, but its rewarding properties contribute to it being the most widely abused illicit drug in the United States (NIDA-SAMHSA, 2014). Currently, half of the United States permit legal provisions for the use of cannabis for assorted therapeutic purposes (i.e. nausea, glaucoma, pain, chemotherapy-induced nausea and vomiting). Recently, Colorado, Washington and the District of Columbia decriminalized the recreational use of marijuana and on November 8th 2016, the state of California will enter a ballot initiative to decriminalize marijuana.

Marijuana contains over 500 identified constituents, and approximately 109 of its constituents are classified as cannabinoids (Mehmedic *et al*., 2010). Δ9-Tetrahydrocannabinol (THC) is the most widely investigated cannabinoid, and is the main psychoactive constituent in marijuana. Other widely investigated cannabinoids include cannabinol (CBN), which was discovered from the Indian hemp at the end of the 19th century (Wood *et al*., 1899) and the non-psychoactive cannabinoid cannabidiol (CBD) (Mechoulam and Gaoni, 1965) and Δ^9-tetrahydrocannabivarin (THCV) (Merkus, 1971) (see table 1). In 1964, the chemical structure of THC was elucidated (Gaoni and Mechoulam, 1964a), which led to a renaissance in the cannabinoid field of research.

The recreational use of marijuana in the 1960s sparked research efforts to investigate the pharmacological and physiological effects of marijuana. Cannabinoids produce a variety of

pharmacological effects in humans and laboratory animals. Collectively, several effects are more unique to cannabinoids than drugs from other classes such as elevated heart rate, ataxia, analgesia, and hypothermia. An early hypothesis to explain the mechanism by which THC produces its effects was that it disrupted neurotransmission by perturbing neuronal cell membranes (Hillard *et al.*, 1985). When evaluating newly synthesized cannabinoids for behavioral activity, a battery of four tests known as the tetrad (hypoactivity, hypothermia, antinociception, catalepsy) is used to distinguish cannabinoids from drugs in different classes (Little *et al.*, 1988), which was later useful in providing functional evidence for a receptor mechanism of action. Also, cannabinoids were evaluated in rodents trained to discriminate THC (Martin *et al.*, 1991), which also provided additional evidence for a receptor mechanism of action.

Following the elucidation of the structure of THC, medicinal chemists developed synthetic cannabinoids (see table 2), which enabled further research to investigate structure activity relationships (SARs). These SAR studies were instrumental in demonstrating that small changes in drug structure dramatically altered drug potency, which greatly supported a receptor mechanism of action. One synthetic cannabinoid, CP 55,940 which was synthesized by Pfizer (Koe *et al.*, 1985) along with other synthetic compounds such as HU-210 (Howlett *et al.*, 1990), and WIN55-212-2 (D'Ambra *et al.*, 1992) helped advance cannabinoid research and understand the mechanisms that generate their physiological effects. Synthetic cannabinoids as well as THC have different binding affinities for the CB_1 and CB_2 receptors that result in differences in their individual potencies. Unlike THC, which is a partial efficacy agonist in vitro, synthetic

cannabinoids produced full agonist like properties (Howlett *et al.*, 1988; Breivogel *et al.*, 1998).

Although these early structure activity relationship studies provided important insights, the specific mechanism of cannabinoids in the brain remained unknown. This gap in our understanding was overcome by the discovery that cannabinoids inhibit adenylyl cyclase activity in model neuronal systems (Howlett and Fleming, 1984; Howlett, 1985). Also, CP 55, 940 (Ki = 25 nM) (Howlett, 1987) was more potent than THC (Ki = 430 nM) at inhibiting adenylyl cyclase (Howlett *et al*., 1988) and was used in the first radioligand binding studies that identified that cannabinoids bind to a specific receptor (Devane *et al*., 1988). In addition, (-)-CP 55, 940 was found to be 200-fold more potent than its positive enantiomer CP 56, 667 at inhibiting adenylate cyclase activity and have higher affinity (Matsuda *et al*., 1990). Also, the inactive enantiomer WIN 55, 212-3 of WIN 55, 212-2 was devoid of pharmacological effects in the tetrad assay (Martin *et al.*, 1991; Compton *et al.*, 1992). These collective findings provided strong evidence that cannabinoids produced their effects by through a receptor mechanism of action.

In combination with the receptor binding data, the tetrad assay was beneficial in determining that CB_1 receptors mediated the central effects of cannabinoids (Compton *et al.*, 1993), by comparing the structural features of cannabinoids with their in vivo activity (Wiley et al., 2014). The pharmacological effects of cannabinoid receptor agonists showed high correlations between the tetrad *in vivo* measures and CB_1 receptor binding affinity, as follows: decrease in spontaneous locomotor activity (r = 0.91), antinociception (r = 0.9), hypothermia (r = 0.89), and catalepsy (r = 0.85) (Compton *et al.*, 1993). For example, nearly 60 different cannabinoids were found to displace [^{3}H] CP 55-940 from its binding site (Compton *et al*.,

1996), and produced tetrad effects that correlated with binding affinity.

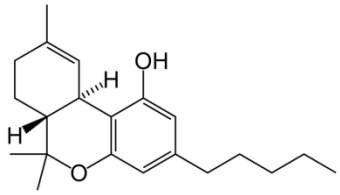
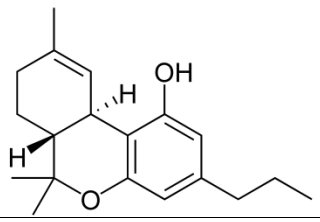
Receptor autoradiography studies led to the discovery that the CB_1 receptor is heterogeneously located throughout the central nervous system (CNS) (Herkenham *et al.*, 1991), and is responsible for the cannabimimetic side-effect profile of marijuana, which includes abuse, dependence, and memory impairment (Lichtman *et al.*, 1995; Hampson and Deadwyler, 1999; Justinova *et al.*, 2003). These effects are produced by activating G-protein coupled CB_1 receptors that inhibit adenylyl cyclase activity and dampen cAMP production (Howlett *et al.*, 1990). In addition, cannabinoid receptor activation attenuates N and P/Q-type calcium channels activity, inhibits cAMP production, and the release of excitatory and inhibitory neurotransmitters. CB_1 receptors are located on presynaptic GABAergic and glutamatergic neurons (Katona *et al.*, 1999) and the stimulation of CB_1 receptors leads to a reduction in the respective neurotransmitters GABA and glutamate.

Using both CB_1 (-/-) mice and pharmacological antagonists of CB_1 receptors (Rinaldi-Carmona *et al.*, 1994; Compton *et al.*, 1996), revealed that the pharmacological effects of THC as well as synthetic cannabinoids in the tetrad are CB_1 receptor mediated. Also, pharmacological antagonists of CB_1 receptors attenuate the behavioral effects of cannabinoids in the tetrad (Long *et al.*, 2009; Blankman and Cravatt, 2013). In addition, these genetic and pharmacological tools were used to show that CB_1 receptor activation can be attributed to several common features of marijuana including increased feeding (Smart *et al.*, 2000), reduced emesis and nausea (Darmani and Pandya, 2000; Darmani, 2001), reductions in pain (Ignatowska-Jankowska *et al.*, 2015; Ghosh *et al.*, 2015) and impairments in memory (Lichtman and Martin, 1996; Niyuhire *et al.*,

2007).

The second major binding site for cannabinoids is the cannabinoid receptor type-2 (CB_2) receptor (Munro *et al.*, 1993). CB_2 receptors are involved in the immune and hematopoietic systems. CB_2 receptor messenger RNA and protein are predominately expressed in microglia (Carlisle *et al.*, 2002) brainstem neurons (Van Sickle *et al.*, 2005; Onaivi *et al.*, 2006) and the periphery (Cabral and Marciano-Cabral, 2005). Also, activation of CB_2 receptors modulates cytokine production (Klein *et al.*, 2003), suppresses the proliferative response of T and B cells to mitogens through the induction of apoptosis (Lombard *et al.*, 2007), and reduces monocyte chemotaxis through PI3K/Akt and ERK1/2 signaling (Montecucco *et al.*, 2008). Finally, CB_1 and CB_2 receptors share approximately 44% homology (Munro *et al.*, 1993).

Table 1. Prevalent phytocannabinoids found in marijuana

Phytocannabinoid	Structure	Reference
Tetrahydrocannabinol (THC)		(Gaoni & Mechoulam, 1964)
Tetrahydrocannabivarin (THCV)		(Merkus, 1971)

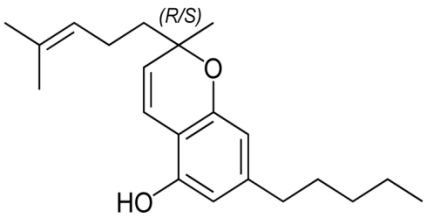
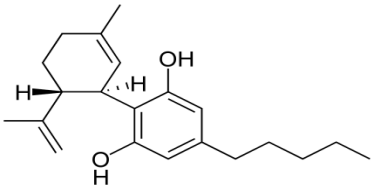
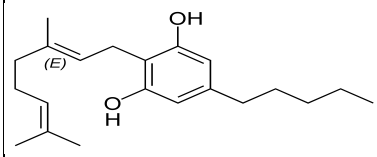
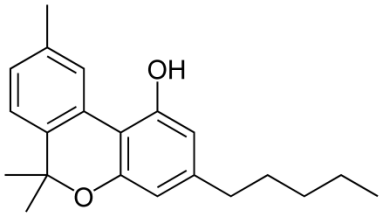
Cannabichromene (CBC)		(Gaoni & Mechoulam, 1966)
Cannabidiol (CBD)		(Michoulam and Shvo, 1963)
Cannabigerol (CBG)		(Gaoni and Mechoulam, 1964b)
Cannabinol (CBN)		(Wood et al., 1899)

Table 2. Synthetic cannabinoids

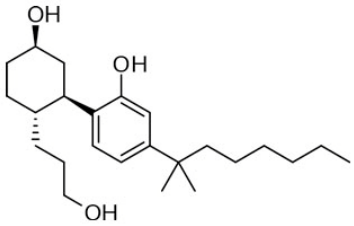
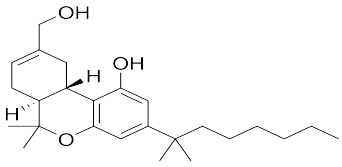
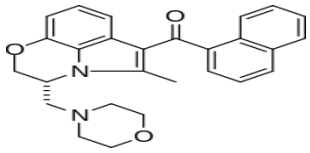
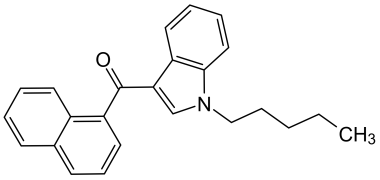
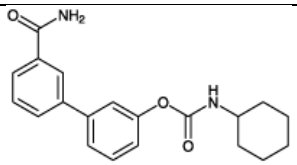
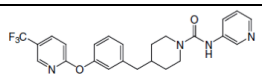
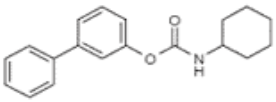
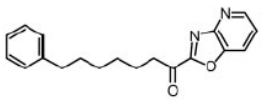
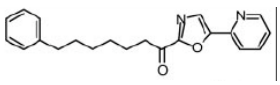
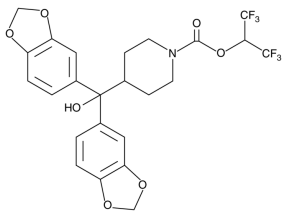
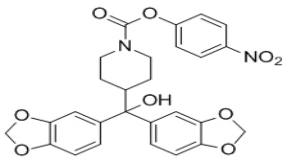
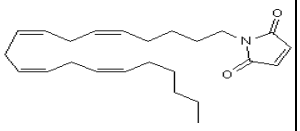
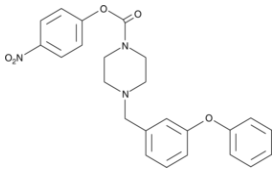
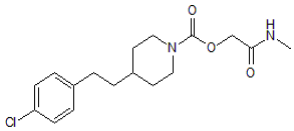
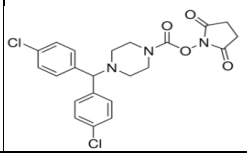
Synthetic Cannabinoid	Structure	Reference
CP 55,940		Koe et al., 1985
HU-210		Howlett *et al.*, 1990
WIN 55-212		D'Ambra et al., 1992
JWH-018		Huffman *et al.*, 1994

Table 3. Endocannabinoid catabolic enzyme inhibitors.

Enzyme Inhibitor	Structure	Reference	Target Enzyme
URB597		(Boger et al., 2005, Piomelli et al., 2006)	FAAH (IC_{50} = 5 nM)
PF-3845		(Ahn et al., 2009)	FAAH (IC_{50} = 230 nM)
URB524		(Mor *et al.*, 2008)	FAAH (IC_{50} = 63 nM)
OL-92		(Boger *et al.*, 2005)	FAAH (IC_{50} = 0.28 nM)
OL-135		(Boger *et al.*, 2005)	FAAH (IC_{50} = 2.1 nM)
KML29		(Chang *et al.*, 2012)	MAGL (IC_{50} = 15 nM)

JZL184		(Labar et al., 2010)	JZL184 (IC_{50} = 8 nM)
N-arachidonylmaleimide		(Labar et al., 2010)	MAGL (IC_{50} = 140 nM)
JZL195		(Long et al., 2009)	FAAH (IC_{50} = 2 nM) MAGL (IC_{50} = 4 nM)
SA-57		(Niphakis et al., 2011)	FAAH (IC_{50} = <10 nM) MAGL (IC_{50} = 410 nM)
MJN110		(Niphakis *et al*., 2013)	MAGL (IC_{50} = 2.1 nM)

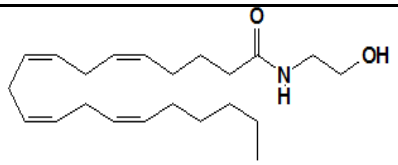
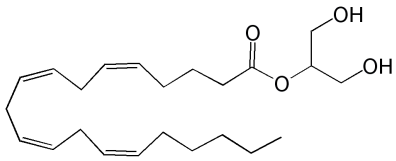
The discovery of endogenous marijuana-like molecules (endocannabinoids) represented a significant breakthrough in cannabinoid research. The first endocannabinoid isolated from porcine brain and identified by mass spectrometry and nuclear magnetic resonance spectroscopy was N-arachidonylethanolamide and was named anandamide (AEA) after the Sanskrit word for bliss (Devane *et al.*, 1992) (see table 4). Anandamide competed with the specific binding of the radiolabeled cannabinoid probe [^{3}H] HU-243. The second endocannabinoid identified was 2-arachidonoylglycerol (2-AG), isolated from canine intestines, and rat brain synaptysomes

(Mechoulam *et al.*, 1995; Sugiura *et al.*, 1995) (see table 4). 2-AG inhibited adenylate cyclase production in mouse spleen, with a similar potency as THC (Mechoulam *et al.*, 1995). Also, intravenous administration of 2-AG produces effects commonly observed with THC in the tetrad assay including immobility, antinociception, reduced spontaneous activity, and decreased rectal temperature. AEA and 2-AG are synthesized on post-synaptic neurons from phospholipids and released on demand (i.e. as needed) and travel in a *retrograde* manner from the post-synaptic neuron terminal to pre-synaptic neuronal CB_1 receptors.

The endocannabinoid (AEA) is synthesized and degraded through distinct biosynthetic and degradative enzymatic pathways. The synthesis of AEA is not completely understood, but one candidate enzyme is NAPE-phospholipase D (NAPE-PLD) (Nyilas *et al.*, 2008) however, NAPE-PLD $^{(-/-)}$ mice do not show reductions in N-acylethanolamines (NAES) (Simon and Cravatt, 2010). After on demand synthesis and release into the synapse, AEA is rapidly degraded in postsynaptic neurons by the enzyme fatty acid amide hydrolase (FAAH) (Cravatt *et al.*, 1996; Giang and Cravatt, 1997; Gulyas *et al.*, 2004). Efforts to determine the molecular identity of the enzyme that degraded AEA were facilitated by a structurally related bioactive lipid oleamide (Cravatt *et al.*, 1995). Anandamide and the sleep-inducing lipids oleamide, had similar hydrolysis activities in N18 neuroblastoma cells (Maurelli *et al.*, 1995). Expression of rat brain oleamide hydrolase confirmed that anandamide was an additional substrate of this enzyme (Cravatt *et al.*, 2001). In addition to AEA, FAAH regulates the levels of other ethanolamides including palmitoylethanolamide (PEA), oleamide, and oleoylethanolamide (OEA) (Cravatt *et*

al., 1995). The endogenous fatty acid amide PEA isolated from soybeans and peanuts was discovered to have anti-inflammatory properties (Kuehl *et al.*, 1957).

Table 4. Endogenous cannabinoids

Endogenous cannabinoid	Structure	Reference
N-arachidonoylethanolamine (AEA)		(Devane et al., 1992)
2-Arachidonoylglycerol (2-AG)		(Mechoulam et al., 1995; Sugiura et al., 1995)

The biosynthesis of neuronal 2-AG is much better understood than the biosynthesis of AEA. The synthesis of 2-AG occurs by the cleavage of diacylglygerol (DAG) by DAG lipase-alpha (DAGLα) (Bisogno *et al.*, 2003) and DAG lipase-beta (DAGLβ). These two enzymes are differentially expressed on cells in the nervous system and peripheral tissue (Hsu *et al.*, 2012). DAGLα is expressed more abundantly than DAGLβ throughout the CNS (e.g. amygdala, cerebellum, hippocampus, frontal cortex, and spinal cord). DAGLα is expressed on postsynaptic neurons in various brain regions (Katona *et al.*, 2006; Yoshida *et al.*, 2006; Lafourcade *et al.*, 2007) and is abundant around dendritic spines which are present in postsynaptic neurons in the cerebellum and hippocampus, and DAGLβ is expressed on macrophages and microglia (Hsu *et al.*, 2012). Also, brain levels of 2-AG are significantly reduced in mice devoid of (DAGLα) compared to mice without (DAGLβ) (Gao *et al.*, 2010; Tanimura *et al.*, 2010).

Several studies implicated MAGL as a key regulator of 2-AG and arachidonic acid levels in CNS. For example, overexpression of MAGL in rat neurons attenuates the accumulation of 2-AG (Dinh et al., 2002). Also, depletion of MAGL in rat brain proteomes decreases 2-AG hydrolysis by 50% (Dinh et al., 2004). The degradation of 2-AG (approximately 85%) is regulated on the presynaptic neuron by MAGL (Blankman et al., 2007). In the rat brain, MAGL is largely expressed in the cerebellum, cortex, thalamus, and hippocampus (Dinh et al., 2002) and is primarily localized to presynaptic terminals (Gulyas et al., 2004). The remaining 2-AG is degraded by the enzymes alpha/beta hydrolase domain 6 and 12 (Blankman et al., 2007). Degradation of 2-AG results in an increase in available arachidonic acid levels in the brain, which is a major source of prostanoids and prostaglandins (Nomura et al., 2011).

Later, the synthesis of pharmacological inhibitors of eCB hydrolysis, and mice devoid of the endocannabinoid regulating enzymes provided abundant research opportunities (see table 3). For example, FAAH inhibitors have proven to have therapeutic potential in a wide variety of pre-clinical assays (Piomelli et al., 2006). The FAAH inhibitor PF-3845 reduces a subset of somatic withdrawal signs in opioid dependent mice (jumps and paw flutters) (Ramesh *et al.*, 2011). The FAAH inhibitor URB597 shows anxiolytic-like activity in the elevated zero maze (Kathuria et al., 2003) and also produces antidepressant effects in forced swim assay (Gobbi et al., 2005). Also, FAAH inhibitors display similar analgesic properties in a variety of animal models of pain (see review)(Schlosburg *et al.*, 2009) and reduces inflammation-induced edema (Cravatt *et al.*, 2004; Holt *et al.*, 2005; Wise *et al.*, 2008). One benefit of FAAH inhibitors is the absence of the full set of cannabimimetic effects or THC-like subjective effects as measured in the tetrad and drug discrimination assays (see section below on cannabinoid drug discrimination).

Genetic deletion of FAAH provided the first evidence that FAAH was the principal hydrolytic enzyme of anandamide (Cravatt *et al.*, 2001). Mice devoid of FAAH have approximately 15 fold-increases in elevations of AEA in the brain of wild-type mice (Cravatt *et al.*, 2001) but retain normal levels of 2-AG (Osei-Hyiaman *et al.*, 2005) and CB_1 expression (Lichtman and Martin, 2002). FAAH $^{(-/-)}$ mice are largely indistinguishable from wild-type mice. Exogenous administration of anandamide produces robust effects in the tetrad (analgesia, hypo-motility, hypothermia, and catalepsy) (Ahn *et al.*, 2008). Also, FAAH regulates the levels of other ethanolamides including palmitoylethanolamide (PEA), oleamide, and oleoylethanolamide (OEA) (Cravatt *et al.*, 1995). One benefit of genetically deleting FAAH over pharmacological

inhibitors is a more direct examination without off-target drug effects, of the physiological role of AEA in the endogenous cannabinoid system. For example, URB597, OL-135, CAY-10402 inhibits FAAH in the CNS but also target peripheral tissues. There is also the added benefit of combining genetic with pharmacological approaches. Specifically, one study using a complementary pharmacological and genetic approach observed that administration of AEA exogenously to animals treated with URB597 or FAAH $^{(-/-)}$ produce tetrad behavioral effects (Cravatt et al., 2001; Fegley et al., 2005), indicating in the absence of FAAH, exogenous AEA can produces effects that are similar to THC.

The development of MAGL inhibitors provided a new approach to prevent 2-AG hydrolysis and understand the physiological properties of 2-AG (see table 4). The MAGL inhibitor JZL184 is able to elevate 2-AG levels 10-fold without elevating AEA (Long, *et al.*, 2009). Previous work in the Lichtman lab observed that the MAGL inhibitor JZL184 attenuated somatic withdrawal signs (i.e. spontaneous jumping, paw flutters, wet-dog shakes) in opioid dependent mice (Ramesh *et al.*, 2011). In contrast to FAAH inhibition, The MAGL inhibitor JZL184 produced increased cannabimimetic effects including antinociception, hypomotility, hyper-reflexia, and partial THC-like subjective effects which are attenuated by the CB_1 antagonist rimonabant and not observed in CB_1 $^{(-/-)}$ mice (Long, *et al.*, 2009). In addition, repeated administration of JZL184 produces pharmacological tolerance and receptor desensitization of CB_1 receptors (Schlosburg *et al.*, 2010). Genetic deletion of MAGL and repeated administration of JZL184 does not retain its analgesic properties and produces cross-tolerance to WIN 55, 212-2 and PF-3845.

MAGL $^{(-/-)}$ knockout mice were developed as a complementary approach to pharmacological inhibition of MAGL. MAGL $^{(-/-)}$ mice have downregulated CB_1 receptors, approximately 90% reductions in enzymatic activity, and about a 10-fold increases in brain 2-AG levels (Schlosburg et al., 2010). Brain levels of arachidonic acid levels are also reduced in MAGL $^{(-/-)}$ mice (Schlosburg et al., 2010). The observation that arachidonic acid levels are reduced in MAGL $^{(-/-)}$ is consistent with observations that that arachidonic acid levels are decreased by organophosphorus agents that inhibit MAGL (Nomura *et al*., 2008). Later, it was discovered that 2-AG is a major endocannabinoid precursor of arachidonic acid in the brain, spleen, lung and liver (Nomura *et al.*, 2008). These findings provided the first insights that the endocannabinoid and eicosanoid signaling pathways might be interconnected.

Investigating the psychoactive effects of cannabinoids

The psychoactive effects of cannabinoids are attributed to the stimulation of neuronal CB_1 receptors. Laboratory assays such as intracranial self-stimulation (ICSS), self-administration (SA), conditioned place preference (CPP), and drug discrimination (DD) are used to investigate the abuse-related effects of drugs (Solinas *et al.*, 2006), and the receptors that contribute to their effects (Balster, 1991; Maldonado, 2002). Attempts to study the abuse-related effects of cannabinoids in ICSS, SA, and CPP have proven very difficult (Maldonado, 2002), and reports indicating that cannabinoids are rewarding or aversive are inconsistent. In the limited number of studies that report reinforcing, or rewarding effects of cannabinoids, the observations are limited

to one species (squirrel monkeys), one assay (self-administration), a specific lab group, or a strict set of experimental parameters (i.e. priming injections in CPP).

In the drug self-administration paradigm, cannabinoids are not readily intravenously self-administered in rodents or non-human primates (Mansbach and Nicholson, 1994) (See Table 1). One explanation is that cannabinoids have a long duration of effects, with maximal plasma concentrations of THC (1000 ng/ml) occurring immediately after exposure to smoke in rhesus monkeys and approximately 80 ng/ml 45 minutes after exposure in humans (Slikker *et al.*, 1991). Commonly in THC self-administration studies, lab subjects are food-restricted before test sessions (Takahashi and Singer, 1979; de la Garza and Johanson, 1987) or administered other drugs of abuse (Tanda *et al.*, 2000). The only consistent observation of intravenously self-administered THC has been in squirrel monkeys (Justinova *et al.*, 2003) in which squirrel monkeys with no history of exposure to any other drugs learned to press a lever for intravenous THC and the acquisition of THC self-administration was very rapid (Justinova *et al.*, 2003). In addition to squirrel monkeys, rats self-administer THC if administered via intracerebroventricular route of administration. In these limited examples, self-administration of THC can be extinguished either by administering vehicle instead of THC, or the CB_1 receptor antagonist SR141716A.

In the ICSS paradigm, animals learn to respond for electrical pulses into medial forebrain bundle, the location in the brain responsible for the rewarding effects of ICSS (See Table 2) (Kornetsky, 1985). Many studies report that drugs of abuse lower electrical stimulation thresholds, which suggests a similar degree of reward can be obtained with less electrical

stimulation in the presence of a known drug that produces rewarding effects (Bauzo and Bruijnzeel, 2012). Cannabinoid ICSS studies have revealed mixed observations. Particularly, some studies reveal that low doses of THC can decrease thresholds for ICSS (Gardner *et al.*, 1988) but others failed to observe facilitation of ICSS with THC (Vlachou *et al.*, 2007; Wiebelhaus *et al.*, 2015). The differences among the studies could be due to the dose, strain of animal, or procedural variations. One example is that high, but not lower doses of cannabinoids are reported to produce anxiogenic effects in mice (Kinden and Zhang, 2015).

The conditioned place preference paradigm is based on the principles of Pavlovian conditioning. This assay involves three phases (habituation, conditioning and testing) in which laboratory subjects are tested in an apparatus with two compartments, where one compartment may contain different floors, environmental and drug-related cues. The other compartment is paired with the drugs vehicle. During the habituation phase, subjects are allowed to explore freely the apparatus before conditioning. During the conditioning phase, an unconditioned stimulus (i.e. drug) is administered to the subject and the animal can explore only one compartment. Sometimes a 3rd neutral chamber is used but is not paired with a drug, and the entrance between both compartments can be opened to allow animal subjects free passage between both chambers. On test days, animals are not administered drug, and the duration of time spent in each chamber is scored. Subjects will voluntarily spend more time in the compartment associated with the drug-related cues, if the drug is rewarding, and the vehicle side if the drug is aversive, and an approximately equal amount of time if the drug is neutral. In the CPP paradigm, cannabinoids are reported to produce both conditioned place preference (CPP)

and conditioned place aversion (CPA) to THC (Parker and Gillies, 1995; McGregor *et al.*, 1996; Chaperon *et al.*, 1998) (See Table 3). Importantly, in most CPP studies, preference or aversive to THC and other cannabinoids are mainly attributed to the dose and time between the injection and the test session. For example, high doses of THC produce CPP in rats if the interval between injection and testing was 24 h. However, if the interval was 48 h, THC produced CPA at high doses (Lepore *et al.*, 1995). It could be that 48 h after injection, mice are undergoing withdrawal and have aversive internal states. In mice, THC induces CPP only when the animals were previously administered a priming injection of THC 24 h before the first conditioning session (Valjent and Maldonado, 2000), although in CD1 mice without A2A adenosine receptors utilizing the same methods (Valjent and Maldonado, 2000) THC produced conditioned place aversion (Soria *et al.*, 2004). These findings indicate the conditioned place preference paradigm does not produce consistent findings to make inferences regarding the rewarding effects of cannabinoids.

In contrast to the above mentioned paradigms, cannabinoids consistently and reliably serve as discriminative stimuli in two-lever or two-aperture drug discrimination studies (Balster and Prescott, 1992; Barrett *et al.*, 1995; Wiley *et al.*, 1995; Burkey and Nation, 1997; Järbe *et al.*, 2001, 2014). Cannabinoids are pharmacologically specific in drug discrimination and only drugs that stimulate CB_1 receptors can fully substitute (produce over 80% of total responses on the lever paired with the training drug) for the discriminative stimulus effects of THC and other cannabinoids. Likewise, the pharmacological specificity of drug discrimination is observed with other classes of drugs (Solinas *et al.*, 2006). Therefore, the drug discrimination paradigm has

proven over time to be the most reliable pre-clinical assay to investigate the psychoactive/subjective properties of cannabinoids.

Table 1 Evaluation of self-administration (SA) of cannabinoids in laboratory animals
SA = Self-Administration

Species	Drug	Dose	ROA	Outcome	Reference
Rhesus monkeys	THC	100–400 μg/kg		No SA	Deneau and S, 1971
Rhesus monkeys	THC	25 - 300 μg/kg		No SA	Deneau and S, 1971
Rhesus monkeys	THC	100–400 μg/kg		No SA	Kaymakcalan, 1973
Rhesus monkeys	THC	17-100 μg/kg		No SA	Pickens *et al.*, 1973
Rhesus monkeys	THC	25 - 300 μg/kg	I.V.	No SA	Harris *et al.*, 1974
Rhesus monkeys	THC	3-300 μg/kg	I.V.	No SA	Carney *et al.*, 1977
Wistar Rat	THC	7.5-300 μg/kg	I.V.	Partial self-admin	Ree *et al.*, 1978
Rat	THC	6.25 - 50 μg/kg	I.V.	Increase SA	Takahashi and Singer, 1979
Rhesus monkeys	CP 55, 940	0.3–3 μg/kg	I.V.	No SA	Mansbach and Nicholson, 1994
Rhesus monkeys	THC		I.V.	No SA	Mansbach and Nicholson, 1994
ICR mice	Win 55,212-2	10–500 μg/kg	I.V.	Increase SA	Martellotta *et al.*, 1998
Squirrel Monkey	THC	1 - 16 μg/kg	I.V.	Increase SA	Tanda *et al.*, 2000
Squirrel Monkey	Win 55,212-2	6.25 - 50 μg/kg	I.V.	Increase SA	Fattore *et al.*, 2001
Wistar Rat	Win 55,212-2		I.V.	Increase SA	Fattore *et al.*, 2001
Wistar Rat	CP 55,940	0.1 - 1.6 mg/2μ1	I.C.V	SA	Braida et al. 2001
Squirrel Monkey	THC	2 - 8 mg/kg	I.V.	Increase SA	Justinova *et al.*, 2003
Wistar Rat	THC	0.01-1 μg/kg	I.C.V	Increase SA	Braida *et al.*, 2004
Squirrel Monkey	AEA	40 mg/kg	I.V.	Increase SA	Justinova et al. 2005
Squirrel Monkey	Methanandamide	10 - 40 μg/kg	I.V.	Increase SA	Justinova et al. 2005
Sprague-Dawley Rat	Win 55,212-2	12.5 μg/kg	I.C.V	SA	Lecca et al. 2006

Lister Hooded Rat	Win 55,212-2	12.5 μg/kg	I.V.	Increase SA	Fadda et al. 2006
Lister Hooded Rat	Win 55,212-2	12.5 μg/kg	I.V.	SA	Fattore et al. 2007
Squirrel Monkey	2 - AG	0.1 - 100 μg/kg	I.V.	Increase SA	Justinova et al. 2011
Sprague-Dawley Rat	2 - AG	25 mg/kg	I.V.	Increase SA	De Luca MA et al. 2014
Long - Evans rats	Win 55,212-2	0.1 mg/kg	I.V.	SA	Lefever TW et al. 2014
Squirrel Monkey	AM404	10 μg/kg	I.V.	SA	Schindler et al. 2016

Table 2 Evaluation of intracranial self-stimulation of cannabinoids in laboratory animals

Species	Drug	Dose	ROA	Outcome	Reference
Long-Evan Rat	THC, nabilone	0.12-10 mg/kg	P.O.	Attenuates ICSS	Stark and Dews, 1980
Lewis rat	THC	1.5 mg/kg	I.P.	Facilitates ICSS	Gardner *et al.*, 1988
Lewis rat	THC	1 and 1.5 mg/kg	I.P.	Attenuates ICSS	Gardner *et al.*, 1988
Sprague-Dawley rat Lewis Rat	THC	1 mg/kg	I.P.	no effect Facilitates ICSS	Lepore *et al.*, 1996
Lewis rat	CP 55,940	10 - 50 µg/kg	I.P.	no effect	Arnold *et al.*, 2001
Sprague-Dawley Rat	SR141716A (CB_1 antagonist)	1 - 10 mg/kg	I.P.	Attenuates ICSS	Deroche-Gamonet *et al.*, 2001
Sprague-Dawley Rat	WIN 55,212-2	0.1 – 1 mg/kg	I.P.	Attenuates ICSS	Vlachou *et al.*, 2003
Sprague-Dawley Rat	URB-597 (FAAH inhibitor) SR141716A	0.3 - 3 mg/kg 0.02 mg/kg	I.P.	Attenuates ICSS	Vlachou *et al.*, 2006
Sprague-Dawley Rat	THC	1 - 2 mg/kg	I.P.	Attenuates ICSS	Vlachou *et al.*, 2007
Sprague-Dawley Rat	THC	0.5 - 1 mg/kg	I.P.	no effect	Fokos and Panagis, 2010
Sprague-Dawley Rat	WIN 55,212-2	0.1 - 1 mg/kg	I.P.	Attenuates ICSS	Mavrikaki *et al.*, 2010
Sprague-Dawley Rat	THC	0.1 mg/kg 1 mg/kg	I.P.	Facilitates ICSS Attenuates ICSS	Katsidoni *et al.*, 2013
C57bl/6 mice	THC JZL184 (MAGL inhibitor) PF3845 (FAAH inhibitor) SA-57	5.6 - 10 mg/kg 16 - 40 mg/kg 30 mg/kg 3 - 17.8 mg/kg	N/A	Attenuates ICSS	Wiebelhaus *et al.*, 2015
C57bl/6 mice	CP 55,940	0.12 - 0.18 mg/kg	s.c.	Attenuates ICSS	Grim *et al.*, 2015

Table 3 Evaluation of conditioned place preference (CPP) of cannabinoids in laboratory animals
CPA = conditioned place aversion; CPP = conditioned place preference

Species	Drug	Dose	ROA	Outcome	Reference
Long-Evans Rat	THC	1, 2 & 4 mg/kg	I.P.	CPP	Lepore *et al*, 1995
Sprague-Dawley rats	THC	10 mg/kg	I.P.	CPA	Parker and Gillies, 1995
Sprague-Dawley Rat	THC	15 mg/kg	I.P.	CPA	Sañudo-Peña *et al.*, 1997
Wistar Rat	WIN 55,212-2	1 mg/kg	S.C.	CPA	Chaperon *et al.*, 1998
ICR mice	THC	20 mg/kg	I.P.	CPA	Hutcheson *et al.*, 1998
Wistar Rat	THC	1-1.5 mg/kg	I.P.	CPA	Mallet and Beninger, 1998
Lister hooded Rat	THC	1.5 mg/kg	I.P.	CPA	Cheer *et al.*, 2000
ICR mice	THC	5 mg/kg	I.P.	CPA	Valjent and Maldonado, 2000
ICR mice	THC	1 mg/kg	I.P.	CPP	Valjent and Maldonado, 2000
Wistar rat	CP 55, 940	20 μg/kg	I.P.	CPP	Braida *et al.*, 2001
Wistar rat	THC	0.075-0.75 mg/kg	I.P.	CPP	Braida *et al.*, 2004
Rat	AM-404 (endogenous cannabinoid reuptake inhibitor)	1.25-10mg/kg	I.P.	CPP	Bortolato *et al.*, 2006
Sprague-Dawley Rat	THC	0.1 mg/kg	I.P.	CPP	Le Foll *et al.*, 2006
C57bl/6	THC	0, 1 & 3 mg/kg	I.P.	no effect	Vlachou *et al.*, 2007
ICR mice	THC	10 mg/kg	I.P.	CPA	Vann *et al.*, 2008
Sprague-Dawley Rat	Win 55,212-2	0.1-1 mg/kg	I.P.	no effect	Polissidis *et al.*, 2009
Sprague-Dawley Rat	JWH-175	0.1 mg/kg	I.P.	CPP	Tampus *et al.*, 2015

Overview of cannabinoid drug discrimination

In the drug discrimination paradigm, laboratory subjects learn to discriminate a drug from its vehicle based on Pavlovian and Skinnerian principles of learning. This principle of learning indicates behavior that is reinforced tends to be repeated and behavior that is not reinforced will be extinguished. In drug discrimination studies, animal behavior such as nose pokes or lever presses are reinforced by the presentation of a positive reward (i.e. food pellet). In drug discrimination procedures, laboratory subjects are trained over time to discriminate the subjective effects of a drug from its vehicle control. After successful training, subjects will typically press a lever, or poke their nose in an aperture for reinforcement (i.e. generally food) inside or on the lever or aperture that is paired with the training drug or its vehicle. After successful acquisition of a discriminative stimulus, novel drugs can be tested to determine if the subjective effects of the training drug and the test drug overlap. If responses occur on the same lever or aperture as the training drug, it is interpreted that the training drug and test drug produce an overlapping internal stimulus. Usually, test and training drugs will produce overlapping internal stimuli if both drugs bind the same receptors (Wiley 1999; Solinas *et al.*, 2006).

In contrast, the interpretation of a partially overlapping discriminative stimulus (i.e. partial substitution) continues to be an ongoing subject of debate in the drug discrimination field (Solinas *et al.*, 2006). For example, researchers in one lab speculate that partial generalization of opioids depends on the types of opioid receptors that are activated and the level of intrinsic activity (Colpaert, 1988). However, if a training drug activates multiple receptors, a test drug that activates only one of these receptors might be discriminated by some, but not all laboratory

subjects. If the data is averaged, it would appear as a partially overlapping discriminative stimulus, but there might not be any individual subject showing a partial effect (Solinas *et al.*, 2005). Moreover, another study tested whether adenosine A1 receptor antagonists substitute for the stimulus effects of caffeine and observed that an A1 antagonist partially overlap with the caffeine discriminative stimulus and that half of the rats produced all responses on the lever paired with drug and the other half produce no responses on the aperture paired with drug (Solinas *et al.*, 2005).

Drugs within the following classification (i.e. nicotinics, hallucinogens, serotonergics, amphetamine-related stimulants, benzodiazepines, aminotetralines, MDA, MDMA, cannabinoids, opiates, inhalants) serve as reliable discriminative stimuli (Barrett and Appel, 1989; Stolerman and Mariathasan, 2003; Solinas *et al.*, 2004). Amphetamine is thought to act as an indirect agonist by releasing dopamine and norepinephrine in the brain (Tseng *et al.*, 1976). Amphetamine serves as a discriminative stimulus and dopaminergic agonists substitute for the amphetamine discriminative stimulus (Young and Glennon, 1986). Also, cocaine, a dopaminergic re-uptake inhibitor substitutes for the amphetamine discriminative stimulus (Goudie, 1991). In addition to stimulants, many drug discrimination studies have been conducted using the anxiolytics. For example, in rats have been trained to discriminate oxazepam and diazepam, but the anxiolytic drug buspirone does not substitute in rats trained to discriminate oxazepam nor diazepam (Hendry *et al.*, 1983). Later, it was discovered that buspirone binds 5-HT receptors, which is distinct from benzodiazepines such as oxazepam and diazepam, which produce their effects on GABA receptors. These observations indicate that laboratory subjects

can discriminate between drugs from different classes, drugs that produce similar internal states (i.e. anxiolytic) and between drugs that produce their subjective effects through distinctly different receptors.

Cannabinoid drug discrimination studies have been conducted for several decades. Several species of animals have been used to study the subjective effects of cannabinoids in drug discrimination. Unlike other assays that are used to investigate drug psychoactivity (i.e. CPP, ICSS, or SA), cannabinoid drug discrimination studies are highly consistent in the observation that a drug can serve as a discriminative stimulus. In early studies, pigeons were trained to discriminate THC by pecking a key to receive food reinforcement (Henriksson *et al.*, 1975; Järbe *et al.*, 1977; Järbe and Hiltunen, 1987). Pigeons are highly sensitive to the subjective effects of cannabinoids (ability to discriminate low doses), although they are rarely used today in drug discrimination studies. In pigeons, THC does not substitute for psychomotor stimulants (Järbe, 1982, 1984), which indicates that the subjective effects of psychomotor stimulants are distinctly different from THC. In rats and mice, different training doses of THC have served as discriminative stimuli (0.25 - 3 mg/kg) (Henriksson *et al.*, 1975; Järbe and McMillan, 1980). Also, genetic approaches employing FAAH $^{(-/-)}$ mice have become available to understand the role of FAAH and AEA in the endocannabinoid system (Vann *et al.*, 2009; Ignatowska-Jankowska *et al.*, 2015). One very important connection between pre-clinical drug discrimination and human drug discrimination studies is that pre-clinical studies are a very good predictor of drug psychoactivity in humans. (Jones and Stone, 1970; Waskow *et al.*, 1970; Fabian *et al.*, 1983; Chait *et al.*, 1988). This highlights a major advantage of the drug discrimination paradigm,

which is the high degree of sensitivity, specificity, and cross-species consistencies in the subjective effects of drugs, in particular, cannabinoids.

Currently, there are no examples that the discriminative stimulus effects of a test drug can completely substitute for the discriminative stimulus effects of THC without activating central CB_1 receptors. For example, rimonabant blocks the discriminative stimulus effects of THC and synthetic cannabinoids, but not SR140098, a CB_1 antagonist that does not cross the blood-brain barrier. However, anti-psychotic drugs (i.e. clozapine, haloperidol, thioridazine, and chlorpromazine) produce all four measures assessed in the tetrad asssay (i.e. catalepsy, antinociception, hypothermia, hypolocomotion) which is a highly predictive assay to screen for CB_1 receptor activity (Wiley, 2003). These observations of cannabinoids in both the drug discrimination and tetrad assay indicate that drug discrimination paradigm is the more pharmacological and behaviorally selective assay for screening the cannabimimetic effects of drugs. Early THC discrimination studies reported cannabidiol, a non-psychoactive cannabinoid, did not substitute for the discriminative stimulus effects of THC (Järbe, 1989; Balster and Prescott, 1992). In addition, drugs that are not considered cannabinoids (i.e. ketamine, alcohol, or cocaine) did not substitute for THC in mice even at very high doses (McMahon *et al.*, 2008). However, several studies observed that diazepam, a benzodiazepine, which does not bind CB_1 receptors, produces average responses that are consistently above 40% for the aperture/lever paired with THC (Mokler *et al.*, 1986; Balster and Prescott, 1992; Wiley and Martin, 1999). CB_1 receptors are located on glutamatergic neurons, and activating CB_1 receptors on glutamatergic neurons can reduce neuronal activity, and may explain these findings, further highlighting the

high degree of sensitivity and selectivity of drug discrimination. Interestingly, CB_1 receptors do not contribute to the partial substitution of diazepam for THC because this effect is attenuated by the benzodiazepine antagonist flumazenil, and not by the CB_1 receptor antagonist rimonabant (Wiley and Martin, 1999).

Finally, cross-substitution of drugs is an important concept in cannabinoid discrimination because it occurs within drugs of the same class, and through a shared mechanism of action. In combination with receptor antagonist studies, cross-substitution studies can provide strong evidence supporting the involvement of a specific receptor mechanism of action of a discriminative stimulus. Cross substitution is observed among many different cannabinoids (Barrett et al., 1995). For example, FAAH $^{(-/-)}$ mice have been trained to discriminate Δ^9-THC, and AEA fully substitutes for THC in FAAH $^{(-/-)}$. However, AEA is rapidly degraded in FAAH $^{(+/+)}$ mice and dose not substitute for THC, suggesting that in the presence of FAAH, AEA cannot produce similar subjective effects as THC. Previous studies have shown that synthetic cannabinoids such as CP 55, 940 and WIN 55,212-2 dose-dependently substitute for THC and cross-substitutes for THC (Wiley, 1999; McMahon et al., 2008). In addition, the synthetic cannabinoid JWH-018 serves as a discriminative stimulus in rhesus monkeys (Ginsburg *et al.*, 2012). THC and JWH-073 substitutes for JWH-018, but GABA receptor agonists such as the benzodiazepines do not substitute for JWH-018 (Rodriguez and McMahon, 2014). One important observation is that when two different drugs fully substitute for each other (cross substitution), it usually occurs through the same receptor mechanism of action. In one reported, mice were trained to discriminate a high dose of methanandamide (70 mg/kg), a drug known to

bind several receptors (i.e. TRPV1, PPAR α) in addition to the CB_1 receptor. In mice trained to discriminate methanandamide (70 mg/kg) the discriminative stimulus was partially attenuated (60% mAEA-like responses) by a large dose of rimonabant (30 mg/kg) (Wiley *et al.*, 2011). Also, a high dose of THC (60 mg/kg) approached full substitution (70% THC-like responses) in mice trained to discriminate methanandamide (70 mg/kg). The results from this set of experiments (Wiley *et al.*, 2011) indicate that higher doses of THC and mAEA have similar but not completely overlapping subjective effects. Given the results in this study, it is plausible that higher doses of mAEA can produce subjective effects through multiple receptors (i.e. CB1, TRPV1) and the subjective effects of THC are produced through only one receptor (i.e. CB_1). Because cross substitution was not observed at higher doses (only partial substitution), these findings validate the idea that cross substitution usually occurs if the mechanism that produces the subjective effects of two different drugs are exactly the same.

Discriminative Stimulus Properties of Cannabinoid Antagonists

The synthesis of the first antagonist of the CB_1 receptor (rimonabant) allowed investigators to determine the role of this receptor in cannabinoid discrimination (Rinaldi-Carmona, 1994). Rimonabant attenuates the discriminative stimulus effects of THC in pigeons, rats, and mice (Wiley *et al.*, 1995; Mansbach *et al.*, 1996; Pério *et al.*, 1996). Antagonist studies in combination with cross-substitution observations with other cannabinoids provide strong evidence that CB_1 receptors are largely responsible for cannabinoid discrimination.

Early reports found that rimonabant fails to serve as a discriminative stimulus using food as a reinforcer (Pério *et al.*, 1996). Interestingly, Rhesus monkeys can learn to discriminate rimonabant if given chronic administration of THC before training sessions (Stewart and Mcmahon, 2010), and discontinuation of chronic THC results in a higher number of responses on the rimonabant associated lever (Stewart and Mcmahon, 2010). Pre-treatment with THC, AEA, CP 55, 940 or WIN 55, 212-2 before rimonabant on test sessions resulted in responses on the vehicle-paired lever and not the rimonabant paired lever, suggesting these other cannabinoids replace the internal subjective effects produced in the absence of chronically administered THC (Stewart and Mcmahon, 2010). Interestingly, the CB_1 antagonist AM251 substituted for discriminative stimulus effects of rimonabant (McMahon, 2006). The above findings may indicate CB_1 antagonists on their own do not produce subjective effects, but they may induce an internal state of withdrawal, that may serve as a discriminative stimulus. Additionally, it is possible that CB_1 antagonist produces a non-drug state.

Discriminative Stimulus Properties of Phytocannabinoids

The most commonly investigated cannabinoids in marijuana (i.e. phytocannabinoids) are THC, cannabidiol (CBD) (Michoulam and Shvo, 1963) cannabinol (CBN) (Wood *et al.*, 1899) cannabichromene (CBC) (Gaoni and Mechoulam, 1966), Δ^9-tetrahydrocannabivarin (THCV) (Merkus, 1971). THC and CBN are the only phytocannabinoids in marijuana that are reported to substitute for the discriminative stimulus effects of THC (Browne and Weissman; Järbe and

Hiltunen, 1987). However, some reports suggest the non-psychoactive cannabinoid CBD can inhibit the discriminative stimulus effects of THC (Pertwee, 2008; Vann *et al.*, 2008).

THC discrimination was used in early studies to screen for THC-like subjective effects of phytocannabinoids (i.e. CBN and CBD), and their metabolites *(11-OH-THC; 8ß-OH-Δ^9-THC; 8α-OH-Δ^9-THC; 8α,11 di-OH-Δ^9-THC, and 8ß,11 di-OH-Δ^9-THC)* in several different species (pigeon, gerbil, rodent, non-human primates, human). CBD is generally thought to have no psychoactive properties on its own, and does not substitute for the discriminative stimulus effects of THC (Järbe and Hiltunen, 1987; Vann *et al.*, 2008). In addition, drug discrimination was used to investigate the subjective effects of inhaled marijuana (Marshell *et al.*, 2014b). When marijuana is inhaled, many constituents (i.e. phytocannabinoids) can interact and different studies have investigated the discriminative stimulus effects of interacting phytocannabinoids. Cannabinoid drug discrimination can also been used to rank order the potency of the subjective effects of phytocannabinoids, and indicates the CBN stimulus is less potent than THC, but the combination of CBN and THC increases the percentage of responses for THC (Järbe and Hiltunen, 1987; Järbe *et al.*, 2014). Thus, THC discrimination has been a very useful to investigate the phytocannabinoids in marijuana that may contribute to the subjective effects of smoked marijuana.

Discriminative Stimulus Properties of Endogenous Cannabinoids

Our understanding of the discriminative stimulus properties of endocannabinoids cannabinoids has been limited until recently because endocannabinoids are rapidly degraded by FAAH and MAGL. Accordingly, early attempts to discover if the subjective effects of AEA overlapped with THC were limited in success (Deutsch and Chin, 1993). In one study, intraperitoneal injections of AEA substituted for the discriminative stimulus effects of THC and CP 55,940 in rats, but not mice, and only at doses that drastically suppressed response rates (Wiley *et al.*, 1995; Wiley *et al.*, 2014), indicating the rapid hydrolysis of AEA prevented an overlapping stimulus in both species.

The synthesis of metabolically stable AEA analogues presented an opportunity to overcome the challenge of rapid degradation. Although, AEA analogues are distinctly different molecules from endogenous (AEA), they have some similarities in their structure. Methylations at carbon 1 and 2 on AEA prevent degradation without significant alterations in affinity or behavioral activity (Adams *et al.*, 1995). For example, (R)-methanandamide, a metabolically stable analogue of AEA dose-dependently substituted for the THC (2 mg/kg) discriminative stimulus in rats (Burkey and Nation, 1997). Interestingly, (R)-methanandamide only produces full substitution in rats that discriminate lower dose of THC (≤ 3.0 mg/kg) (Järbe *et al.*, 1998). (R)-methanandamide substituted partially or not at all in rats trained to discriminate 5.6 or 30 mg/kg THC (Järbe et al., 1998, 2000; Wiley et al., 2011). Although methanandamide is

considered a cannabinoid (Ki = 28.3 ± 3) (Goutopoulos *et al.*, 2001), it also binds TRPV1 receptors that are involved in anandamide-induced reductions in locomotion (de Lago et al., 2004). This observation offers the possibility that methanandamide produces a discriminative stimulus at higher doses that could be mediated by TRPV1 receptors, and lower doses is mediated by CB_1 receptors. These findings indicate that lower doses of THC that produce weaker subjective effects can generate overlaping with the subjective effects with (R)-methanandamide. It is possible that (R)-methanandamide is less potent than THC, or is metabolized before it can occupy the same number of CB_1 receptors. Also, THC is more potent than both O-1812 and 2-methylarachidonyl-2'-fluoroethylamide (analogues of AEA) in mice trained to discriminate O-1812, and substitutes for THC to a greater degree than exogenous AEA in rats and monkeys (Wiley *et al.*, 1997, 2004). These observations indicate differences in the intrinsic activity between various AEA analogues and THC, or that their discriminative stimulus effects occur through a separate receptor mechanism. More illuminating, the CB_1 receptor antagonist rimonabant (0.3 and 1 mg/kg) completely attenuates the ability of (R)-methanandamide (doses ≤ 30 mg/kg) to occasion the lever paired with THC in rats (Järbe *et al.*, 2001). However, extremely high doses of methanandamide (≥ 70 mg/kg) failed to substitute in mice trained to discriminate a high dose of THC (30 mg/kg), and rimonabant did not block the generalization of methanandamide in mice trained to discriminate a high dose of methanandamide (70 mg/kg). This raises the possibility that a non-CB_1 receptor mechanism generates the discriminative stimulus effects of methanandamide (70 mg/kg) (Wiley *et al.*, 2011).

Although short half-lives of the endocannabinoids make it difficult to investigate their effects, FAAH and MAGL inhibitors preventing the rapid hydrolysis of AEA and 2-AG, provide tools to prevent their rapid hydrolysis and understand their general pharmacological properties. Complete blockade of FAAH produces large increases of AEA in mouse brain (Fegley *et al.*, 2005; Ahn *et al.*, 2009; Long *et al.*, 2009; Niphakis *et al.*, 2012). Exogenous administration of AEA does not undergo rapid hydrolysis after FAAH inhibition and produces physiological effects. For example, the FAAH inhibitor URB597 (0.3 mg/kg) or AEA (10 mg/kg) alone does not substitute for THC in Sprague-Dawley rats, however, co-administration of URB597 and i.v. AEA (3 mg/kg) completely substitutes for THC (Solinas *et al.*, 2007). These findings suggest that low doses of exogenously administered AEA are sufficient to produce a THC-like discriminative stimulus if its primary hydrolytic enzyme is inhibited. Moreover, FAAH $^{(-/-)}$ mice successfully learn to discriminate both AEA and THC, and cross-substitution occurs with THC and AEA in FAAH $^{(-/-)}$ mice, indicating in the absence of FAAH, AEA can produce internal subjective states that are similar to THC (Walentiny *et al.*, 2011). The cross-substitution of THC and AEA in FAAH $^{(-/-)}$ mice was attenuated by the CB_1 receptor antagonist rimonabant, indicating a shared contribution of CB_1 receptors in the AEA and THC discriminative stimulus (Walentiny *et al.*, 2011, 2015).

Most drug discrimination research with endocannabinoids has focused on AEA instead of 2-AG. There is one study (Wiley, *et al.*, 2014) in which 2-AG was evaluated in mice trained to discriminate THC and 2-AG did not substitute for THC (Matuszak *et al.*, 2009). Genetically modified mice, specifically MAGL$^{(-/-)}$ mice have not been evaluated in a drug discrimination

procedure, but several pharmacological inhibitors of MAGL activity have been investigated. The MAGL inhibitor JZL184 partially substitutes for THC in wild-type mice, and rats trained to discriminate THC from vehicle (Long *et al.*, 2009; Walentiny *et al.*, 2015). However, one report indicates JZL184 produced responses on the lever paired with vehicle in mice trained to discriminate THC (Hruba *et al.*, 2015). Regardless, MAGL inhibition does not produce an overlaping discriminative stimulus with THC.

Simultaneous blockade of FAAH and MAGL can be obtained by several approaches which include administering mice the dual FAAH and MAGL inhibitors JZL195 or SA-57 (Long *et al.*, 2009; Niphakis *et al.*, 2012; Hruba *et al.*, 2015), and co-administering mice selective FAAH and MAGL inhibitors (Long *et al.*, 2009; Ghosh *et al.*, 2015), or administering MAGL inhibitors in $FAAH^{(-/-)}$ mice (Long *et al.*, 2009). Dual inhibition produces robust cannabimimetic effects including antinociception, hypomotility, hyper-reflexia, catalepsy (Long *et al.*, 2009), and a completely overlapping THC-like discriminative stimulus that are mediate by CB_1 receptors (Long *et al.*, 2009; Hruba *et al.*, 2015). The analgesic effects of dual inhibition are greater than the effects generated by single enzyme inhibition, and catalepsy is only observed after inhibiting both enzymes.

Taken together, these observations indicate the discriminative stimulus/subjective effects of AEA in the presence of FAAH can partially overlap with THC, or completely overlap with THC in the absence of FAAH. Additionally, inhibiting 2-AG degradation can produce increase responses for lever/aperture paired with THC. These observations could be due to 2-AG acting as a full CB_1 agonist, while AEA acts as a partial CB_1 agonist (Sugiura *et al., 2002*) indicating 2-

AG produces greater intrinsic effects at CB_1 receptors than AEA. Elevated levels of 2-AG may achieve greater occupancy of CB_1 receptors in the brain than AEA because bulk brain levels of 2-AG are approximately three orders of magnitude higher than AEA (Ahn *et al.*, 2009; Long *et al.*, 2009).

Table 4 Evaluation of discriminative stimulus properties of cannabinoids in laboratory animals

Species	Training Drug	Training Dose	Substitution Drug	ROA	Outcome	Response rates	Reference
Pigeon	THC	.15 - .20 mg/kg	THC	I.M.	Complete Generalization	Decrease	(Henriksson *et al.*, 1975)
Sprague Dawley Rat/Pigeon	THC	Rats = 3 mg/kg Pigeon = 1 mg/kg	SP-111 11-OH-Δ9-THC 11-OH-Δ8-THC	I.M. = 90 min I.P. = 30 min	Complete Generalization	Decrease	(Järbe and McMillan, 1980)
Sprague-Dawley Rat Rhesus Monkey	THC	3 mg/kg	THC CP 55,940	I.P. in rats I.M. in Monkey	Generalization More potent than THC	Decrease	Gold *et al.*, 1992
Sprague-Dawley Rat Rhesus Monkey	THC	3 mg/kg 1 mg/kg	THC THC	I.P.	Complete Generalization	Decrease	Wiley *et al.*, 1993
Sprague-Dawley Rat	CP 55,940	0.1 mg/kg	THC WIN 55,212-2	I.P.	Complete Substitution Complete substitution	Decrease	Wiley *et al.*, 1995

Sprague-Dawley Rat	WIN 55,212-2	0.3 mg/kg	CP 55,940 THC Rimonabant	S.C.	Complete Substitution Complete Substitution Complete Antagonism		Pério *et al.*, 1996
Sprague-Dawley Rat	R-Methanandamide (AEA analogue) THC	10 mg/kg 3 mg/kg	SR141716A Anandamide	I.P.	Complete Antagonism Complete Substitution	Decrease	Järbe *et al.*, 2001
Wistar Rat	CP 55,940	0.03 - 0.014 mg/kg	THC Rimonabant	I.P.	Complete Substitution Complete Antagonism	No effect	De Vry and Jentzsch, 2003
Sprague-Dawley Rat	THC	3.2 mg/kg	Anandamide R-Methanandamide	I.P.	Partial Substitution Complete Substitution	Decrease	Alici and Appel, 2004
Sprague-Dawley Rat	THC	3 - 10 mg/kg	B - endorphin SR141716A	I.P.	Potentiate Generalization of THC Complete Attenuation	Decrease	Solinas *et al.*, 2004
Sprague-Dawley Rat	THC O-1812 (CB_1 agonist)	3 mg/kg 0.3 mg/kg	O-1812 THC	I.P.	Complete Substitution Complete Substitution	Decrease	Wiley *et al.*, 2004
Sprague-Daley Rat	THC R-Methanandamide	1.8 - 5.6 mg/kg 10 mg/kg	Rimonabant AM251	I.P.	Complete Antagonism	No effect	Järbe *et al.*, 2006

Rhesus Monkey	THC	0.32 mg/kg	Rimonabant AM251	I.V.	Complete Antagonism	Statistics not reported	McMahon, 2006
Rhesus Monkey	THC	0.1 mg/kg	CP 55,940 WIN 55,212-2 R - Methanandamide Rimonabant	I.V.	Complete substitution Complete substitution Complete substitution Complete Antagonism	Decrease	McMahon, 2006
Sprague-Dawley Rat	THC	3 mg/kg	I.V.Anandamide I.V. Anandamide + URB597	I.V.	Complete substitution	Decrease	Solinas *et al.*, 2007
Sprague-Dawley Rat	AM1346 (AEA analogue)	3 mg/kg	AM1346 mAEA	I.P.	Complete substitution Complete substitution Complete substitution	AM1346 = no change mAEA = decrease	Järbe *et al.*, 2009
C57BL/6 mice	THC	10 mg/kg	JWH-202 JWH-204 JWH-205	S.C.	No substitution Complete substitution Complete substitution	JWH-205 = decrease THC = decrease	Vann *et al.*, 2009
C57BL/6 mice	THC	5.6 mg/kg	THC JZL195 JZL184	I.P.	Complete Generalization Complete substitution Partial substitution	Statistics not reported	Long *et al.*, 2009

FAAH $^{(-/-)}$ mice	THC	5.6 mg/kg	THC JZL195 JZL184	I.P.	Complete Generalization Complete substitution Complete substitution	Statistics not reported	Long *et al.*, 2009
Sprague-Dawley Rat	THC	1.8 mg/kg	WIN 55,212-2	I.P.	Complete substitution	Statistics not reported	Järbe *et al.*, 2010
Sprague-Dawley Rat	mAEA	10 mg/kg	mAEA THC	I.P.	Complete substitution Complete substitution	Statistics not reported	Järbe *et al.*, 2010
FAAH $^{(-/-)}$ mice	Anandamide	6 mg/kg	THC	I.P.	Complete substitution	Decrease	Walentiny *et al.*, 2011
C57BL/6 mice	THC	30 mg/kg	AEA methanandamide THC CP 55,940	I.P.	No substitution No substitution Complete substitution Complete substitution	Decrease	Wiley *et al.*, 2011
C57BL/6 mice	methanandamide	70 mg/kg	methanandamide THC	I.P.	Complete Generalization No substitution	Decrease	Wiley *et al.*, 2011
Sprague-Dawley Rat	THC	3 mg/kg	AM598 AM678 AM2233 WIN55212-2	I.P.	Complete substitution Complete substitution	Statistics not reported	Järbe *et al.*, 2011

Rhesus Monkey	THC	0.1 mg/kg	JWH-018 JWH-073	I.V.	Complete substitution	Statistics not reported	Ginsburg *et al.*, 2012
Sprague-Dawley Rat	AM2389 (CB_1 agonist)	0.18 mg/kg and 0.56 mg/kg	THC AM5983	I.P.	Complete substitution	Statistics not reported	Järbe *et al.*, 2012
C57BL/6 mice	THC	5.6 mg/kg	UR-144 XLR-11	I.P.	Complete substitution	Decrease	Wiley *et al.*, 2013
C57BL/6 mice	THC	5.6 mg/kg	KML29	S.C.	No substitution	No effect	Ignatowska-Jankowska *et al.*, 2014
C57BL/6 mice FAAH $^{(-/-)}$ mice	THC AEA	5.6 mg/kg 6 mg/kg	Org27569 + Veh Org27569 + AEA	I.P.	No substitution	No effect	Gamage *et al.*, 2014
Sprague-Dawley Rat	THC JWH-018	3 mg/kg 0.3 mg/kg	JWH-018 THC	I.P.	Complete substitution Complete substitution	Decrease	Wiley *et al.*, 2014

Sprague-Dawley Rat	THC	3 mg/kg	JWH-018 JWH-073 JWH-200 JWH-203 JWH-250 AM-2201 CP 47,497	I.P.	Complete substitution Complete substitution Complete substitution Complete substitution Complete substitution Complete substitution	JWH-073 = Decrease CP 47,497 = Decrease	Gatch and Forster, 2014
C57BL/6 mice	THC	5.6 mg/kg	JZL184 + PF-3845 SA-57 JZL195	I.P.	Complete substitution Complete substitution Complete substitution	Statistics not reported	Hruba *et al.*, 2015
C57BL/6 mice	CP 55,940	0.1 mg/kg	MJN110 JZL184	I.P.	Complete substitution Complete substitution	MJN110 = increase	Ignatowska-Jankowska *et al.*, 2015
C57BL/6 mice	CP 55,940 AEA	0.1 mg/kg 6 mg/kg	ZCZ-011 + CP 55,940 ZCZ-011 + AEA	I.P.	Augments substitution	No effect	Ignatowska-Jankowska *et al.*, 2015

Sprague-Dawley Rat	THC	3 mg/kg	ADB-PINACA THJ-2201 RCS-4 JWH-122 JWH-210	I.P.	Complete substitution Complete substitution Complete substitution Complete substitution Complete substitution	JWH-210 = decrease MDMA = decrease RCS-4 = decrease	Gatch and Forster, 2016
Sprague-Dawley Rat	THC	3 mg/kg	AM2201	I.P.	Complete substitution	Statistics not reported	Järbe *et al.*, 2016

Rationale and Hypothesis

Overall Hypothesis

The overall hypothesis of this dissertation is that an inhibitor of the primary endocannabinoid regulating enzymes FAAH and MAGL will serve as a discriminative stimulus via a CB_1 receptor mechanism of action, and inhibiting both FAAH and MAGL are necessary to generate the discriminative stimulus.

Selection of SA-57

The dual FAAH-MAGL inhibitor SA-57 was selected as the training drug in this dissertation research because it is one of two available pharmacological inhibitors (i.e. SA-57, JZL195) of the endocannabinoid regulating enzymes FAAH and MAGL. SA-57 has the added benefit over JZL195 because it is more potent for FAAH than MAGL (Niphakis *et al.*, 2012), which provides a tool to examine the consequences of inhibiting FAAH with varying degrees of inhibiting MAGL. In addition, SA-57 produces around (10-fold) elevations of AEA and 2-AG in the brain and inhibits FAAH (IC_{50} = 1-3 nM) and MAGL (IC_{50} = 10 μM). At low doses (≤ 1mg/kg) SA-57 produces maximum AEA elevation and at higher doses it incrementally elevates 2-AG. This allows us to investigate the dose-related effects of full FAAH inhibition (i.e., maximized increases in endogenous AEA levels in brain) combined with incremental increases of 2-AG.

Chapter 2. Characterization of the SA-57 discriminative stimulus

To test the hypothesis that the FAAH and MAGL inhibitor SA-57 would serve as a novel discriminative stimulus, we employed the drug discrimination paradigm. First, we trained C57BL/6J mice to discriminate CP 55,940, and then FAAH $^{(-/-)}$ mice trained to discriminate AEA in order to select a training dose of SA-57. Then, we administered SA-57 in a double alternation schedule to determine if SA-57 could be discriminated from its vehicle. We anticipated SA-57 would serve as a discriminative stimulus because the dual FAAH and MAGL inhibitor JZL195 fully substitutes for THC (Long *et al.*, 2009) and elicits cannabimimetic effects, as assessed in the tetrad assay (Long *et al.*, 2009; Anderson *et al.*, 2014; Ghosh *et al.*, 2015), as well as impaired performance in a Morris water maze spatial memory task (Wise *et al.*, 2012). We continued our characterization of the SA-57 discriminative stimulus by conducting a time course study. We anticipated maximum responding for SA-57 would occur at approximately 2h, which corresponds to maximal brain levels of AEA and 2-AG. Finally, we sought to determine if CB_1 receptors were required for the SA-57 discriminative stimulus. In order to determine the receptor mechanism of action, mice were administered the CB_1 receptor antagonist rimonabant.

The second major goal of this dissertation was to determine if inhibiting both FAAH and MAGL in combination, or inhibiting FAAH or MAGL separately was necessary to generate the SA-57 discriminative stimulus. To test this hypothesis, we investigated each enzyme targets of SA-57 (FAAH, MAGL, ABHD6) to delineate the contribution of each enzyme. First we administered the dual inhibitor, JZL195 to determine if the subjective effects of different dual FAAH and MAGL inhibitors would overlap. We expected JZL195 to substitute for SA-57

because both inhibitors completely block FAAH and MAGL activity, and fully elevate AEA and 2-AG. Next we administered two selective FAAH inhibitors (PF3845 and URB597) to test whether FAAH inhibition alone, would substitute for SA-57. Then, we administered two selective MAGL inhibitors (JZL184 and MJN110) determine whether MAGL inhibition alone, would substitute for SA-57. These studies revealed that MAGL inhibitors but not FAAH did in fact fully substitute for SA-57. Also, we employed rimonabant to determine whether CB_1 receptors mediate these effects. Furthermore, we sought to determine if the substitution of MAGL inhibitors was mediated through CB_1 receptors. Finally, because FAAH inhibition elevates other lipids in addition to AEA (i.e., PEA and OEA) and AEA also binds TRPV1 and PPARα receptors (Lo Verme *et al.*, 2005) we employed a selective receptor antagonist of each receptor to investigate their role in the SA-57 discriminative stimulus.

Chapter 2. Characterization of the SA-57 discriminative stimulus

Introduction

Cannabinoid CB_1 (Devane *et al.*, 1988; Matsuda *et al.*, 1990) and CB_2 receptors (Munro *et al.*, 1993) and their endogenous ligands N-arachidonoyl ethanolamine (anandamide; AEA) (Devane *et al.*, 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam *et al.*, 1995; Sugiura *et al.*, 1995) represent primary elements of the endocannabinoid system. This system modulates many physiological processes, including pain (Hohmann *et al.*, 2005; Kinsey *et al.*, 2010; Woodhams *et al.*, 2012; Ignatowska-Jankowska *et al.*, 2014), memory (Hampson and Deadwyler, 1999), appetite (Kirkham and Tucci, 2006), and reward (Tsou *et al.*, 1998; Marsicano and Lutz, 1999). The primary psychoactive constituent of *Cannabis*, Δ^9-tetrahydrocannabinol (THC) (Gaoni and Mechoulam 1964), produces its psychotomimetic effects through CB_1 receptors (Huestis *et al.*, 2001), and induces dopamine release in the nucleus accumbens (Chen *et al.*, 1991), though to a substantially lower magnitude than other abused drugs. Curiously, THC produces reinforcing effects in some (Gardner *et al.*, 1988; Lepore *et al.*, 1996; Justinova *et al.*, 2003, 2005), but not all (Vlachou *et al.*, 2007; Wiebelhaus *et al.*, 2015) preclinical laboratory animal models. In contrast, THC serves as a reliable discriminative stimulus in the drug discrimination paradigm (Henriksson *et al.*, 1975; Järbe, 1989; Wiley *et al.*, 1997; Vann *et al.*, 2009), an assay that is highly predictive of drug psychoactivity in humans (Chait *et al.*, 1988; Kamien *et al.*, 1993; Lile *et al.*, 2012).

Whereas THC elicits relatively long-lasting pharmacological effects, AEA and 2-AG produce short-lived effects because of rapid hydrolysis by their respective primary catabolic enzymes fatty acid amide hydrolase (FAAH) (Cravatt *et al.*, 1996, 2001) and monoacylglycerol lipase (MAGL) (Di Marzo *et al.*, 1999; Dinh *et al.*, 2002). Accordingly, inhibitors of these enzymes elevate endocannabinoid brain levels, and represent useful investigative tools. Although the selective FAAH inhibitors URB597 (Fu *et al.*, 2005) and PF-3845 (Ahn *et al.*, 2009) elevate AEA brain levels and produce antinociceptive effects, neither compound substitutes for THC (Gobbi *et al.*, 2005;Wiley *et al.*, 2014). Similarly, the MAGL inhibitor JZL184 elevates endogenous 2-AG brain levels and produces antinociception, but only partially substitutes for THC (Long et al. 2008; Long et al. 2009; Wiley et al. 2014; Walentiny et al. 2015). Conversely, the dual FAAH-MAGL inhibitor JZL195 fully substitutes for THC, elicits a constellation of cannabimimetic effects (Long *et al.*, 2009; Wise *et al.*, 2012; Hruba *et al.*, 2015) and produces an increased magnitude of antinociceptive effects compared with single enzyme inhibition (Long *et al.*, 2009; Ghosh *et al.*, 2015). Similarly, the dual FAAH-MAGL inhibitor SA-57 fully substitutes for THC in wild-type mice (Hruba et al. 2015).

As it has yet to be established whether an inhibitor of endocannabinoid hydrolysis can serve as the training drug in drug discrimination procedures, the present study investigated whether mice will learn to discriminate SA-57 from vehicle. SA-57 inhibits FAAH much more potently than it inhibits MAGL or ABHD6, another serine hydrolase that degrades 2-AG, but to a much less extent than MAGL (Blankman *et al.*, 2007). Thus, SA-57 possesses utility to investigate the consequences of maximally elevating brain AEA levels, while dose-dependently

increasing brain 2-AG levels (Niphakis *et al.*, 2012). To select the SA-57 training dose, initial experiments examined its dose-effect relationship to substitute for the potent CB_1 receptor agonist CP 55,940 in C57BL/6J mice and AEA in FAAH (-/-) mice (to prevent rapid hydrolysis). Having established that mice learn to discriminate SA-57 from vehicle, we then assessed its dose-response relationship and time course. Because various substrates of FAAH (e.g., AEA, palmitoylethanolamide (PEA), and oleoylethanolamide (OEA)) and MAGL (e.g., 2-AG) bind CB_1, CB_2, TRPV1(Smart *et al.*, 2000), and peroxisome proliferator-activated receptor-alpha (PPARα) receptors (Lo Verme *et al.*, 2005), we tested whether antagonists for these receptors would block the discriminative stimulus effects of SA-57. Additionally, we conducted an extensive series of drug substitution tests to gain further insight into the training dose of the SA-57 discriminative stimulus. Specifically, we tested whether CP 55,940, as well as the non-cannabinoid psychoactive drugs nicotine and diazepam would substitute for the SA-57. As MAGL also plays a rate limiting role in the production of arachidonic acid and prostanoids in brain (Nomura *et al.*, 2011), we examined whether the COX-2 inhibitor valdecoxib, which reduces prostanoid synthesis but does not affect brain endocannabinoid levels, would substitute for SA-57. The final goal of the present study was to elucidate the degree to which relevant endocannabinoid hydrolytic enzyme inhibitors contribute to the SA-57 training dose. Accordingly, we investigated whether individual FAAH, MAGL, and ABHD6 inhibitors, we well as simultaneous inhibition of FAAH and MAGL would substitute for SA-57.

Materials and methods

Subjects

Male C57BL6/J mice (Jackson Laboratory; Bar Harbor, ME) and male FAAH (-/-) mice served as subjects. The FAAH (-/-) mice were backcrossed >14 generations on to a C57BL6/J background. The mice were 9-11 weeks of age at the beginning of training and were individually housed in a temperature-controlled (20-22°C) vivarium in accordance with Virginia Commonwealth University Institutional Animal Care and Use Committee guidelines. Mice were given water ad libitum, and were food restricted to 85-90% of free-feed body weight, which was established during a two-week period of ad libitum food every six months.

Drugs

SA-57, MJN110, KT182, KT195, and JZL195 were synthesized in the Cravatt laboratory, as previously described (Long, Nomura, *et al.*, 2009; Niphakis *et al.*, 2012, 2013; Hsu *et al.*, 2013). N-arachidonoyl ethanolamine (AEA) was provided by Organix Inc. (Woburn, MA), and valdecoxib was provided by Sigma-Aldrich (Saint Louis, MO). CP 55,940, JZL184, PF-3845, rimonabant, and SR144528 were generously supplied by the National Institute on Drug Abuse (NIDA) (Rockville, Maryland, USA). Capsazepine was purchased from Cayman Chemical, and GW6471 was purchased from Tocris Bioscience. Each compound was dissolved in a vehicle consisting of ethanol, emulphor-620 (Rhodia, Cranbury, New Jersey, USA), and saline in a ratio

of 1:1:18. All injections were given via the intraperitoneal (i.p.) route of administration in a volume of 10 μl per 1 g of body weight.

Apparatus

Drug discrimination was conducted in eight sound-attenuating operant conditioning boxes (18 x 18 x 18 cm) (MED Associates, St. Albans, VT). Each operant box contained two nose poke apertures, and a food dispenser delivering 14-mg food pellets to a receptacle chamber located between apertures. Computer software (MED-PC® IV, MED Associates, St. Albans, VT) was used to record nose pokes and to control stimulus presentations and food deliveries.

Drug Discrimination Paradigm

Training

Separate groups of mice were trained to discriminate each of the following three training drugs from vehicle. Groups 1 and 2 consisted of C57BL6/J mice (n=8) trained to discriminate CP 55,940, and FAAH (-/-) mice (n=11) trained to discriminate AEA, respectively. The third group of mice consisted of three cohorts of C57BL6/J mice (n=8/cohort) trained to discriminate SA-57 from vehicle. The treatment conditions for each cohort are described below under Testing. The pretreatment times for the training drugs were 120 min for SA-57 and 30 min for CP 55,940 and AEA. During each 15 min training session, both nose poke apertures were available, but only responses into the correct aperture associated with the appropriate training

drug or vehicle resulted in food reinforcement. Each incorrect response reset the response requirement. Injections before training sessions were conducted (Monday-Friday) in a double alternation sequence of drug (SA-57, CP 55,940, or AEA) and vehicle (e.g., vehicle, vehicle, drug, drug).

Testing

Test sessions were scheduled twice per week, with a minimum of 72 h between test days. To be eligible for testing, subjects were required to meet the following three criteria on nine of the previous ten consecutive training sessions: 1) correct completion of the first FR10 (i.e., first 10 consecutive responses into the appropriate aperture); 2) ≥ 80% correct responding; and 3) maintain response rates ≥ 10 responses/min. During the 15-min test sessions, responses in either aperture resulted in the delivery of food reinforcement according to an FR10 schedule of reinforcement, without a limitation on the number of reinforcers earned within a session. Before conducting substitution tests, dose-response tests with SA-57, CP 55,940 or AEA were conducted to characterize their generalization gradients to their respective discriminative stimulus. For time course studies, animals were injected with SA-57 (10 mg/kg) and tested at 0.25, 1, 2, 4, or 8 h after injection. In order to assess whether CB_1 receptors mediated the discriminative effects of SA-57, and the substitution of CP 55,940, MJN110, JZL184, and JZL195, we challenged with the CB_1 antagonist rimonabant (3 mg/kg; Rinaldi-Carmona, 1994). We also examined whether the CB_2 receptor antagonist SR144528 (3 mg/kg; Rinaldi-Carmona et

al., 1998), the TRPV1 receptor antagonist capsazepine (5 mg/kg; Kinsey et al. 2009), and the PPARα receptor antagonist GW6471 (2 mg/kg; Lo Verme et al. 2005) would block the discriminative stimulus effects of SA-57. Each antagonist was administered 15 min prior to injections of 10 mg/kg SA-57. The three cohorts of mice trained to discriminate SA-57 were employed in the following experiments. All cohorts were included in the SA-57 acquisition curve. Cohort 1 was used in the time-course study, the MJN110 (0.25 – 5 mg/kg), KT182 (1 and 2 mg/kg), KT195 (40 mg/kg), valdecoxib (10 mg/kg), and MJN110 (2.5 mg/kg) + PF3845 (10 mg/kg) substitution studies; cohort 2 was used to test the psychoactive non-cannabinoid drugs nicotine (1.5 mg/kg) and diazepam (10 mg/kg), and in substitution tests with JZL195 (2-20 mg/kg), JZL184 (4-100 mg/kg), PF3845 (10 and 30 mg/kg), and URB597 (10 mg/kg); and cohort 3 was used in the receptor antagonist experiments (rimonabant, SR144528, capsazepine, GW6471).

[^{3}H] SR141716A binding assay

Cerebella were dissected from adult male ICR mice, stored at -80°C, and membranes were prepared as described previously (Selley *et al.*, 2004). Membrane protein (15 μg) was incubated with 0.94 nM [^{3}H] SR141716A in assay buffer (50 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$ and 0.2 mM EGTA) with 0.5% (wt./vol) bovine serum albumin (BSA) in the presence and absence of 5 μM unlabeled SR141716A to determine non-specific and specific binding, respectively. The assay was incubated for 90 min at 30°C and terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filters that were pre-soaked in Tris buffer

containing 0.5% (wt./vol) BSA (Tris-BSA), followed by five washes with cold Tris-BSA. Bound radioactivity was determined by liquid scintillation spectrophotometry at 45% efficiency in ScintiSafe Econo 1 scintillation fluid after a 12-h delay.

Data analysis

The percentage of drug appropriate responses and response rates (responses/min) were recorded for each experiment. Full substitution was defined as greater than or equal to 80% nose pokes that occurred into the aperture associated with the training drug. Partial substitution was defined as greater than or equal to 20% and less than 80% nose pokes in the training drug-paired aperture. Less than 20% nose pokes on the drug-paired aperture was defined as no substitution (Solinas *et al.*, 2006). ED_{50} values (and 95% confidence intervals) for generalization or substitution were calculated using least squares linear regression analysis. Behavioral data are depicted as mean ± S.E.M. The data were analyzed using one-way or two-way ANOVA. Dunnett's tests or Bonferroni post hoc analyses were used following a significant ANOVA for the response rate data. GraphPad Prism 6.0 statistical software (Graph Pad Software, Inc., La Jolla, CA) was used for data analysis.

Binding data were determined in triplicate and are reported as specific binding. Each competition dataset was analyzed by one-way ANOVA to determine concentration-dependence. Rimonabant competition curves were analyzed by non-linear regression to determine IC_{50} and Hill coefficients using a four parameter fit with GraphPad Prism 6.0. The IC_{50} values were then converted to K_i values using the Cheng-Prusoff equation.

Results

SA-57 substitutes for CP 55,940 in C57BL/6J mice and AEA in FAAH (-/-) mice

Figure 1 shows that SA-57 fully substituted for CP 55,940 and AEA in mice trained to discriminate each of these drugs. C57BL/6J mice administered either CP 55,940 or SA-57 completely occasioned the discriminative stimulus effects of CP 55,940 (Figure 1A). SA-57 did not affect response rates; however, CP 55,940 significantly reduced response rates [$F(4,55) = 4.7$; $p < 0.01$], with 0.2 mg/kg yielding significant reductions in response rates compared with vehicle (Figure 1B). In FAAH (-/-) mice trained to discriminate AEA (6 mg/kg) from vehicle, SA-57 also fully substituted for AEA (Figure 1C). FAAH (-/-) mice administered AEA (1-30 mg/kg) or SA-57 (1-10 mg/kg) dose-dependently selected the aperture associated with AEA (Figure 1C). Both AEA [$F(4, 50) = 27.5$; $p < 0.001$] and SA-57 [$F(5, 46) = 15.27$; $p < 0.001$] significantly reduced response rates (Figure 1D). The highest doses tested of AEA (i.e., 30 mg/kg) and SA-57 (i.e., 17 mg/kg) significantly depressed response rates compared with vehicle in FAAH (-/-) mice.

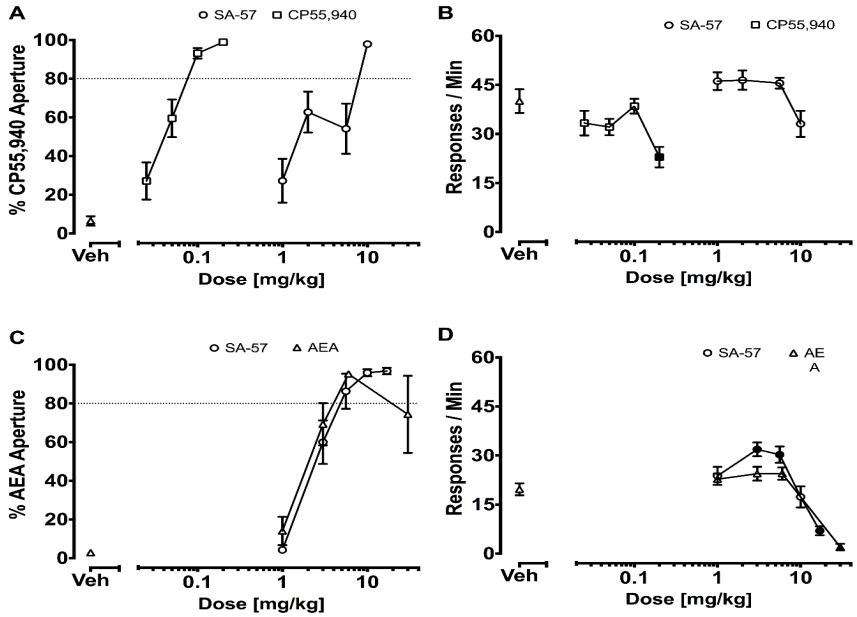


Figure 1. Effects of CP 55,940, AEA and SA-57 on percentage of responses in training drug-paired apertures and response rates in C57BL/6J mice trained to discriminate CP 55,940 (0.1 mg/kg) or FAAH (-/-) mice trained to discriminate AEA (6 mg/kg).

A) Dose-dependent generalization of CP 55,940 and dose-dependent substitution of SA-57 for the CP 55,940 discriminative stimulus. The respective ED50 (95% CI) values for CP 55,940 generalization and SA-57 substitution in C57BL/6J mice were 0.04 (0.03 - 0.05) mg/kg and 2.4 (1.6 – 3.6) mg/kg. B) Respectively, CP 55,940 (0.2 mg/kg), but not SA-57, significantly decreased rates of responding compared to vehicle. C) Dose-dependent generalization of AEA and dose-dependent substitution of SA-57. The respective ED50 (95% CI) values for AEA and SA-57 in FAAH (-/-) mice were 2.7 (2.3-3.1) mg/kg and 3.1 (2.8-3.4) mg/kg. D) SA-57 (17

mg/kg) and AEA (30 mg/kg) decreased rates of responding. Values represent mean ± SEM. Filled symbols indicate significant difference ($p < 0.001$) vs. vehicle; n = 7-10 mice/group.

The SA-57 discriminative stimulus

Because 10 mg/kg SA-57 fully substituted for CP 55,940 in C57BL/6J mice and for FAAH (-/-) mice, this dose of SA-57 was selected as the training dose in three naïve cohorts of mice (n = 8 mice/group). As shown in Figure 2, 50% of mice achieved the criteria to discriminate SA-57 from vehicle by the 27th training session, and 23 of 24 mice acquired the discrimination by day 40. The final mouse achieved criteria on day 74 of training, but was excluded from subsequent experiments because of its substantial delay in acquisition. Similar rates of acquisition were found for CP 55,940 in C57BL/6J mice and AEA in FAAH (-/-) mice.

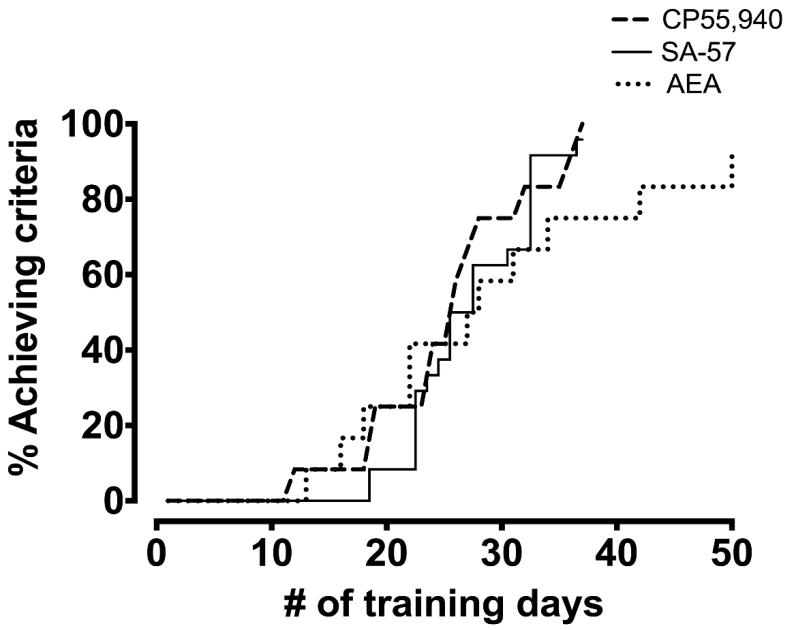


Figure 2. Acquisition rates of SA-57 (10 mg/kg) in C57BL/6J mice, AEA (10 mg/kg) in FAAH (-/-) mice, and CP 55,940 (0.1 mg/kg) in C57BL/6J mice trained in drug discrimination. Values represent percentage of mice that achieved criteria (see text) across days. n = 24 mice for SA57, 12 for AEA, and 12 for CP 55,940.

Figure 3 shows the time course effects of 10 mg/kg SA-57 vs. vehicle for selecting the aperture associated with SA-57 (Figure 3A) and response rates (Figure 3B). Whereas mice that received vehicle responded consistently on the vehicle-associated aperture at each of the time points, mice administered 10 mg/kg SA-57 selected the SA-57 aperture $\geq$ 80% at 1 and 2 h post injection, showed partial substitution at 0.25 and 4 h, and responded predominantly on the vehicle aperture 8 h after injection. There were no differences in rates of responding between mice injected with vehicle or SA-57 at any time point (Figure 3B; $p = 0.48$).

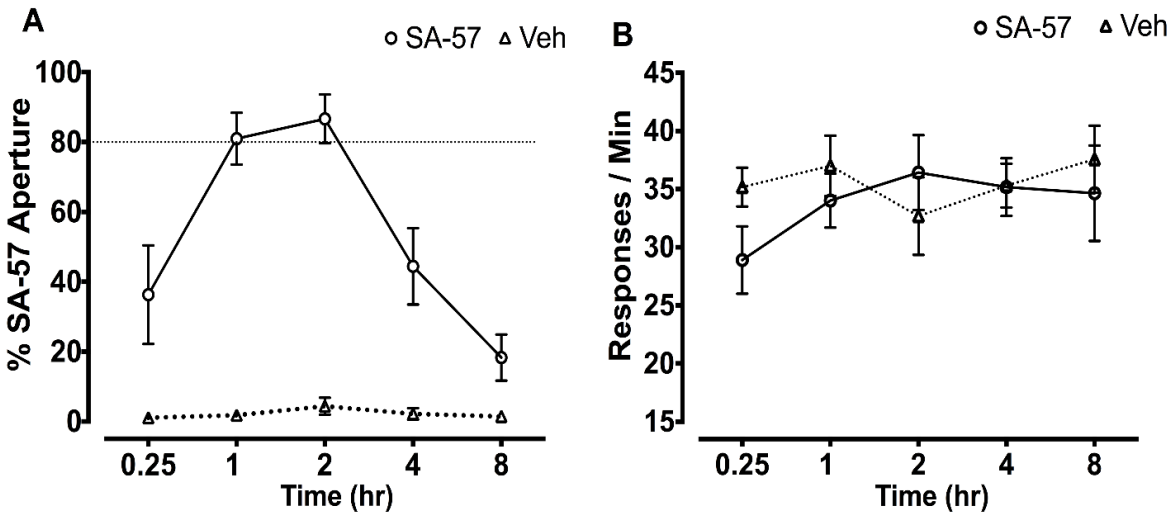


Figure 3. Time course effects for occasioning the 10 mg/kg SA-57 training dose.

A) Percentage of responses in the SA-57-associated aperture 0.25, 1, 2, 4, or 8 h following an injection of vehicle or SA-57 (10 mg/kg). B) SA-57 did not affect response rates at any time point after administration. Values represent mean ± SEM; n = 7 mice/group.

As shown in Figure 4, the CB_1 receptor antagonist rimonabant (0.03-3 mg/kg), significantly blocked the SA-57 training dose. In contrast, the CB_2 receptor antagonist SR144528 (3 mg/kg), the TRPV1 receptor antagonist capsazepine (5 mg/kg), and the PPARα receptor antagonist GW6471 (2 mg/kg) did not block the SA-57 training dose (Table 1).

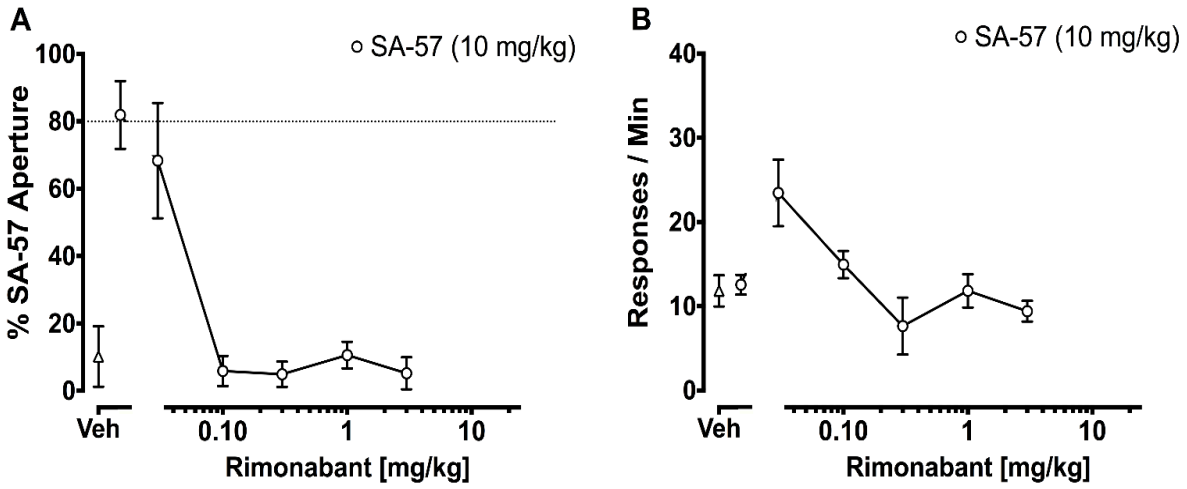


Figure 4. CB_1 receptors play a necessary role in the SA-57 discriminative stimulus.

A) Rimonabant (0.03-3 mg/kg) significantly attenuated the SA-57 training dose. B) Rimonabant doses (i.e., 0.1, 0.3, 1, 3 mg/kg) that blocked the SA-57 training dose did not reduce response rates. Triangles represent vehicle controls, and open circles represent injections of SA-57. Values represent mean ± SEM; n = 3-6 mice/group.

Table 1. CB_1 receptors mediate the discriminative stimulus effects of the SA-57 (10 mg/kg) training dose. The CB_1 receptor antagonist rimonabant (3 mg/kg) significantly blocked the discriminative stimulus effects of SA-57 (10 mg/kg) as well as substitution of CP 55,940 (0.1 mg/kg). The CB_2 receptor antagonist SR144528 (3 mg/kg), the TRPV1 receptor antagonist capsazepine (5 mg/kg), and the PPARα receptor antagonist GW6471 (2 mg/kg) did not block the SA-57 (10 mg/kg) discriminative stimulus. The vehicle-vehicle and rimonabant-vehicle conditions are the same as those used in Figure 9. Values represent mean ± SEM. n = 6-8 mice/group.

Drug	Antagonist	% SA-57 Substitution +/- SEM	Nose Pokes/min +/- SEM
Vehicle	Vehicle	12.8 ± 9.4	38.9 ± 3
	Rimonabant	4.0 ± 1.2	24.9 ± 3
	SR144528	0.7 ± 0.3	36.6 ± 3.8
	Capsazepine	1.3 ± 0.4	20.1 ± 2.6
	GW6471	0.3 ± 2.6	24.6 ± 3.1
SA-57	Vehicle	95.7 ± 1.7	27.3 ± 1.9
	Rimonabant	3.4 ± 1.2	20.1 ± 2.5
	SR144528	98 ± 1.5	30.5 ± 5.4
	Capsazepine	86 ± 12.2	15.7 ± 2.9
	GW6471	96.5 ± 1.3	19.0 ± 2
CP 55,940	Vehicle	82.5 ± 11	33.1 ± 3.3
	Rimonabant	10.4 ± 5.7	20.7 ± 4.9

SA-57 does not bind CB_1 receptors

As the SA-57 discriminative stimulus required CB_1 receptor activation, we next examined whether this compound interacts directly with CB_1 receptors. Accordingly, we tested if SA-57 would displace [^{3}H] SR141716A binding in mouse cerebellar membranes. As shown in Figure 5, rimonabant (i.e., unlabeled SR141716A) inhibited [^{3}H] SR141716A binding in a concentration-dependent manner ($p < 0.001$, $F = 17.36$, $df = 7$), with a K_i value of 0.75 ± 0.16 nM and Hill coefficient of 0.97 ± 0.08. In contrast, SA-57 (0.01 to 10 μM) did not inhibit [^{3}H] SR141716A binding ($p = 0.96$; Figure 5), indicating that this compound does not directly interact with CB_1 receptors.

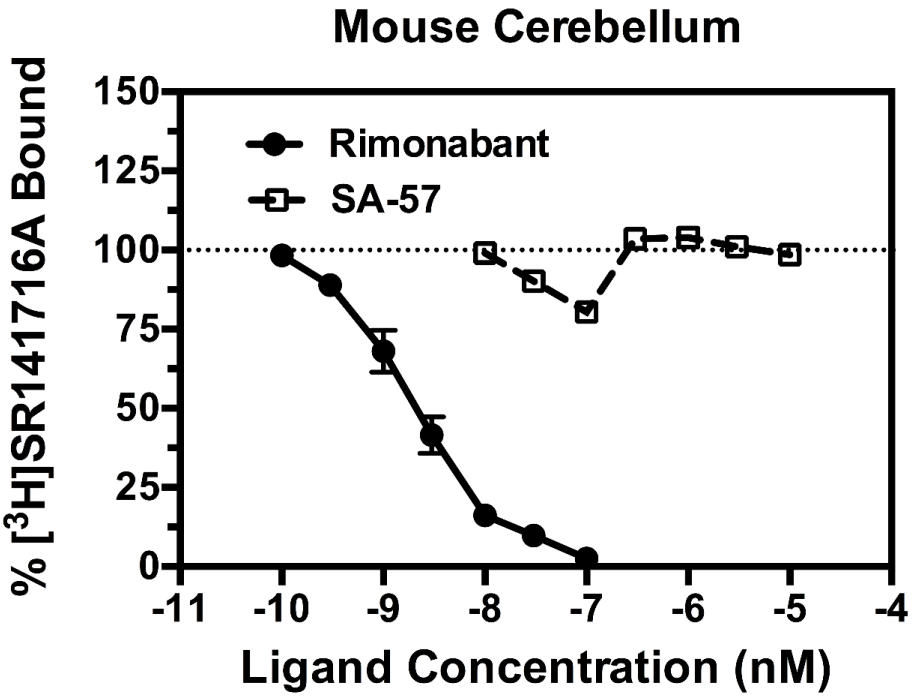


Figure 5. SA-57 does not compete with [3H] SR141716A binding to CB_1 receptors in mouse cerebellum.

Data represent mean [^{3}H] SR141716A bound (pmol/mg) ± SEM in the presence of varying concentrations of rimonabant or SA-57 (n = 3). Specific binding of [^{3}H] SR141716A in the absence of competing ligand was 1.65 ± 0.26 pmol/mg. Similar results were obtained with [^{3}H] CP 55,940 binding in membranes prepared from Chinese hamster ovary cells stably expressing the mouse CB_1 receptor, in which concentrations of up to 10 μM SA-57 did not affect binding (data not shown).

Substitution tests in SA-57 discriminating mice

We next tested whether the non-cannabinoid, psychoactive compounds, nicotine and diazepam, would substitute for SA-57. As shown in Figure 6A, nicotine did not substitute for SA-57, but diazepam produced partial substitution. Both drugs significantly reduced response rates [Figure 6B; $F(3,28) = 14.01$; $p < 0.001$], demonstrating that behaviorally active doses were reached.

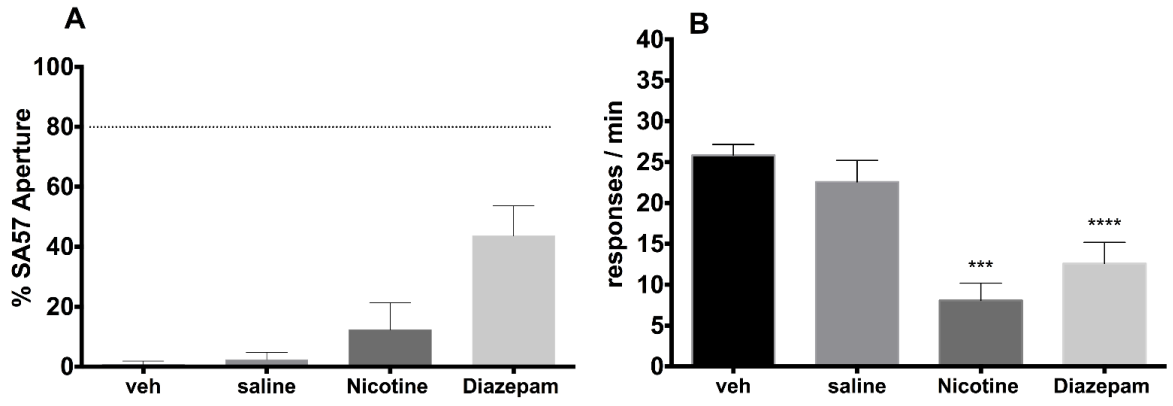


Figure 6. Substitution experiments of non-cannabinoid psychoactive drugs nicotine (1.5 mg/kg) and diazepam (10 mg/kg) for the SA-57 training dose.

A) Nicotine did not substitute, while diazepam partially substituted for the SA-57 training dose. B) Nicotine (1.5 mg/kg) and diazepam (10 mg/kg) significantly reduced rates of responding. Values represent mean ± SEM. Asterisks indicate significant difference ($p < 0.05$) vs. vehicle; n = 7-8 mice/group.

Figure 7 shows the dose-effect curves of the mixed CB_1/CB_2 receptor agonist CP 55,940, the dual FAAH-MAGL inhibitor JZL195, and SA-57 in mice trained to discriminate SA-57 (10 mg/kg) from vehicle. CP 55,940, JZL195, and SA-57 produced dose-related responding into the aperture associated with SA-57 (Figure 7A). CP 55,940 [$F(3,28) = 2.99$, $p < 0.05$] and SA-57 [$F(4,42) = 2.78$, $p < 0.05$], but not JZL195, reduced response rates (Figure 7B).

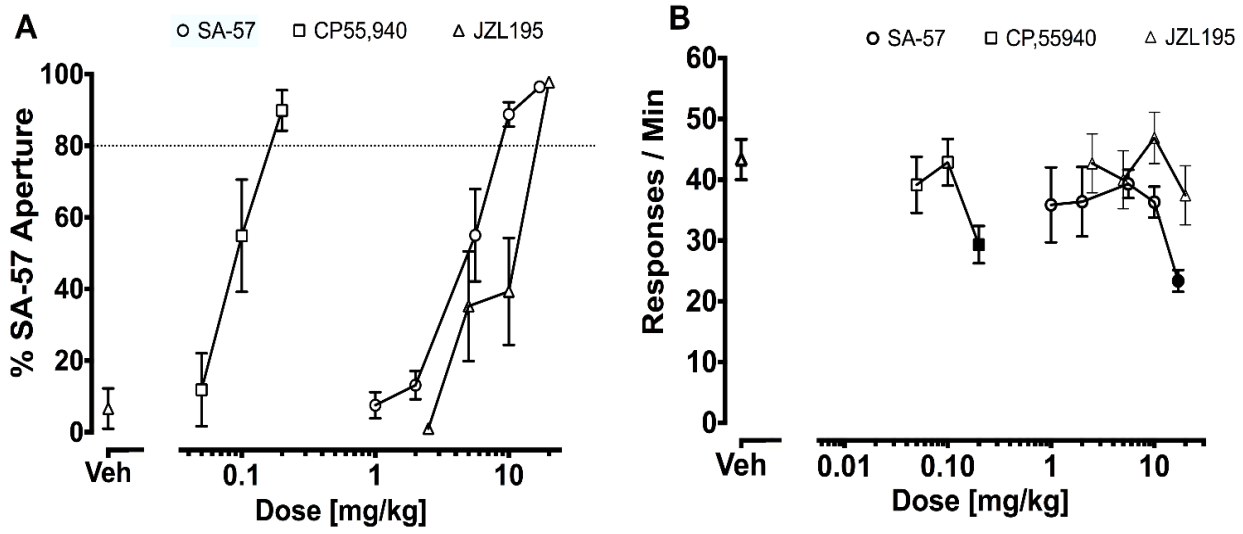


Figure 7. Evaluation of the dose-response relationships of SA-57, CP 55,940, and JZL195 to occasion the SA-57 (10 mg/kg) discriminative stimulus.

A) SA-57 produced dose-dependent generalization, and CP 55,940 and JZL195 dose-dependently substituted for SA-57. The respective ED_{50} (95% CI) values for CP 55,940 substitution, JZL195, and SA-57 generalization were 0.096 (0.076 – 0.121) mg/kg, 6.2 (3.5 – 10.9) mg/kg, and 4.4 (3.5 - 5.4) mg/kg. B) Respectively, doses of CP 55,940 (0.2 mg/kg) or SA-57 (17 mg/kg) significantly reduced response rates. Values represent mean ± SEM. Filled symbols indicate significant difference ($p < 0.05$) vs. vehicle; n = 7-8 mice/group.

SA-57 generalized to itself in a dose-dependent fashion, and the MAGL inhibitors MJN110 and JZL184 dose-dependently substituted for SA-57 (Figure 8A). Although MJN110 did not affect response rates [$F(6, 48) = 0.33$, $p = 0.92$], the highest doses of SA-57 (17 mg/kg) [$F(5, 42) = 3.391$, $p < 0.05$] and JZL184 (100 mg/kg) [$F(4, 18) = 3.985$, $p < 0.05$] significantly reduced response rates (Figure 8B).

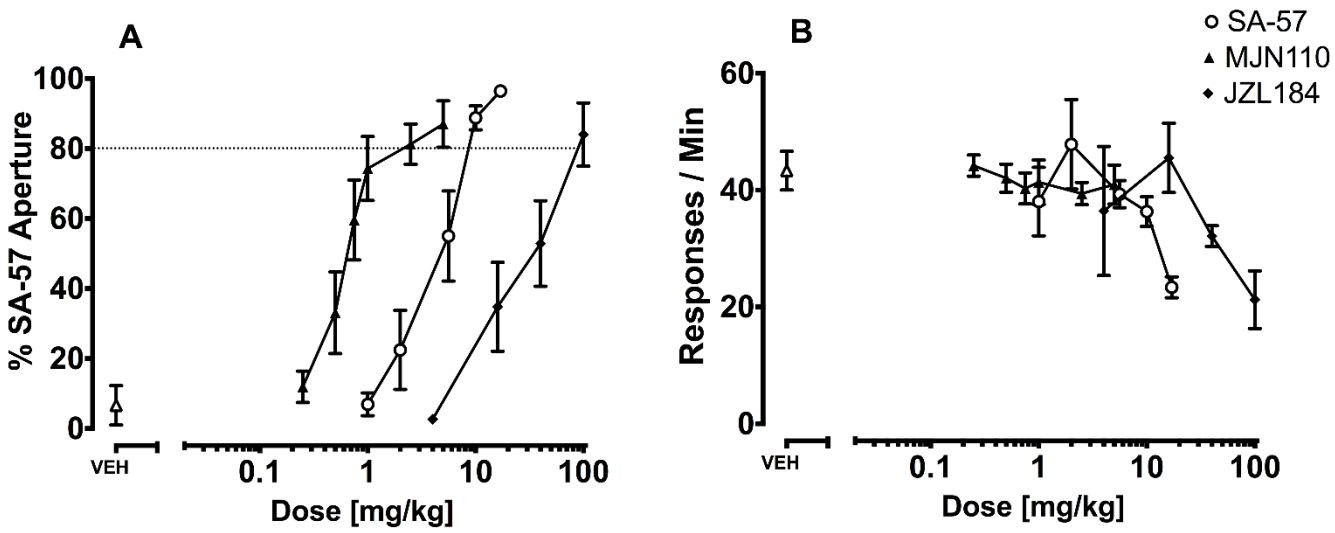


Figure 8. Evaluation of the dose-response relationships of SA-57, MJN110 and JZL184 to occasion the SA-57 (10 mg/kg) stimulus.

A) Dose-dependent generalization of SA-57 and dose-dependent substitution of MJN110 and JZL184. The respective ED_{50} (95% CI) values for MJN110 and JZL184 generalization and SA-57 substitution in C57BL/6J mice were 0.77 (0.53 – 1.1) mg/kg and 20.44 (11 – 37.97) mg/kg, and 4.39 (3.53 – 5.45) mg/kg. B) SA-57 (17 mg/kg) and JZL184 (100 mg/kg), significantly decreased rates of responding. Values represent mean ± SEM. ** indicate significant difference ($p < 0.001$) vs. vehicle; n = 7-8 mice/group.

As shown in Figure 9A, rimonabant (3 mg/kg) completely blocked substitution of MJN110 (5 mg/kg), JZL184 (100 mg/kg), and JZL195 (20 mg/kg) for the SA-57 training dose. Also, rimonabant significantly reduced rates of responding [Figure 9B; $F(1, 29) = 11.91$, $p < 0.01$].

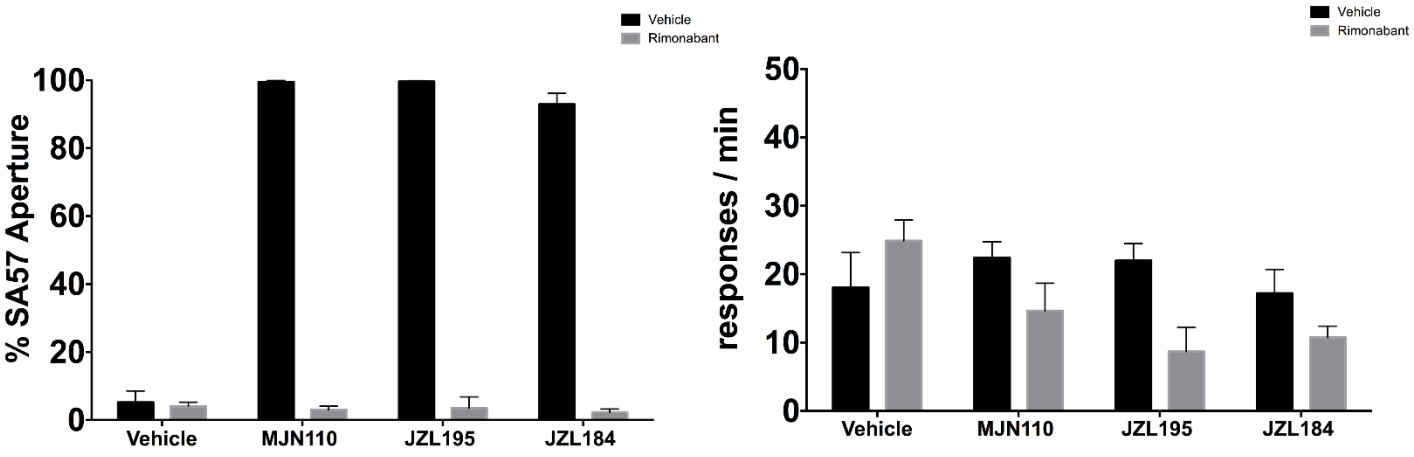


Figure 9. Substitution of MJN110 (5 mg/kg), JZL184 (100 mg/kg) and JZL195 (20 mg/kg) for SA-57 (10 mg/kg) requires CB_1 receptors.

A) Rimonabant (3 mg/kg) completely blocked MJN110, JZL184 and JZL195 substitution. B) Rimonabant did affect response rates. Values represent mean ± SEM; n = 7-8 mice/group.

In contrast, mice administered high doses of the FAAH inhibitors PF-3845 (10 and 30 mg/kg) and URB597 (10 mg/kg) selected the vehicle aperture (Table 5). Likewise, mice given dosesABHD6 inhibitors that completely block enzyme activity, KT182 (1 and 2 mg/kg) or KT195 (40 mg/kg), as well as mice given high dose of the selective COX2 inhibitor valdecoxib (10 mg/kg) selected the vehicle aperture.

Enzyme	Drug (mg/kg)	% SA-57 Substitution +/- SEM	(Nose Pokes/min) +/-SEM
FAAH	Vehicle	0.6 ± 0.5	47.5 ± 4.9
	PF-3845 (10)	1.5 ± 0.5	34.4 ± 3.8
	PF-3845 (30)	0.7 ± 0.2	38.9 ± 3.5
	URB597 (10)	2.1 ± 1.0	36.9 ± 5.6
ABHD6	Vehicle	1.1 ± 0.6	46.5 ± 2.6
	KT182 (1)	1.4 ± 0.7	41.1 ± 3.5
	KT182 (2)	1.4 ± 0.8	43.5 ± 2.4
	KT195 (40)	0.8 ± 0.3	38.1 ± 3.1
COX2	Vehicle	1.1 ± 0.6	46.5 ± 2.6
	Valdecoxib (10)	1.1 ± 0.7	31.9 ± 3.9

Table 5. FAAH inhibitors (PF-3845 and URB597), ABHD6 inhibitors (KT182 and KT195), and the COX2 selective inhibitor valdecoxib do not substitute for the discriminative stimulus effects of SA-57 (10 mg/kg) in C57BL/6J mice and do not affect response rates. Values represent mean ± SEM; n = 7-8 mice/group.

Because MAGL inhibitors, but not FAAH inhibitors, substituted for SA-57, we next examined whether full FAAH inhibition would elicit a leftward shift in the MAGL substitution dose-response curve. Accordingly, we tested the dose-response relationship of MJN110 with PF3845 (10 mg/kg) or vehicle for substitution in mice trained to discriminate SA-57 from vehicle. As shown in Figure 10A, PF-3845 elicited a significant leftward shift in the MJN110 substitution dose-response curve [potency ratio (95% CL) = 1.84 (1.3 – 2.8)] (Colquhoun, 1971). The ED_{50} (95% CI) values for the MJN110 + Veh and MJN110 + PF3845 groups were 0.89 (0.68 – 1.15) and 0.51 (0.27 – 0.95) mg/kg, respectively. No significant changes were found for response rates (Figure 10B).

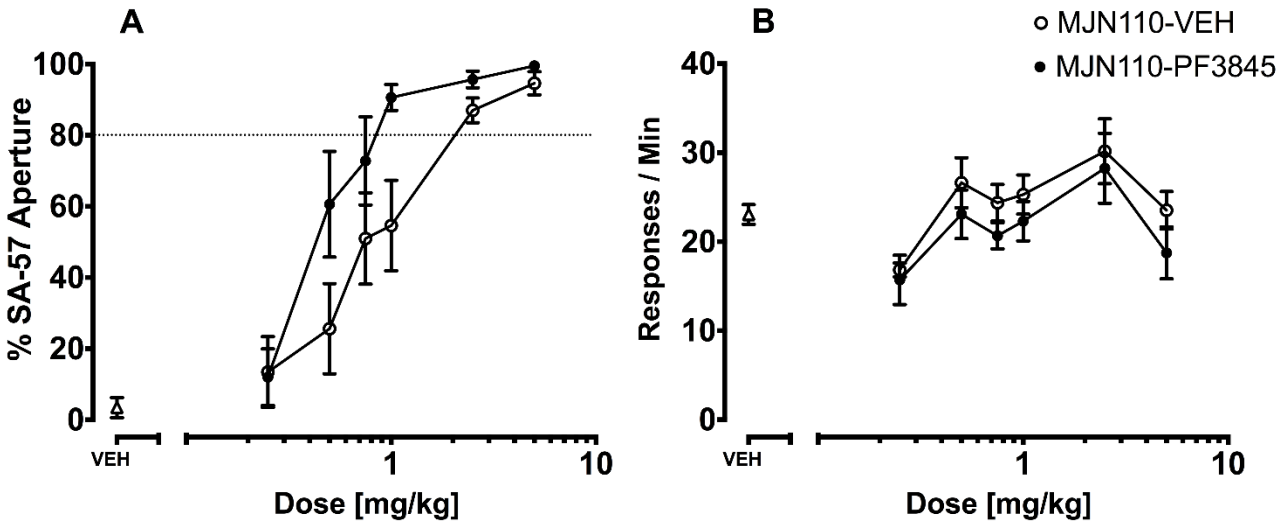


Figure 10. The FAAH inhibitor PF-3845 augments the MJN110 substitution dose-response curve for SA-57 (10 mg/kg).

A) PF-3845 (10 mg/kg) produced a leftward shift of the MJN110 substitution dose-response curve. B) None of the drug combinations significantly decreased rates of responding. Values represent mean ± SEM; n = 7-8 mice/group.

Discussion

The present study demonstrates that mice readily learn to discriminate the dual FAAH-MAGL inhibitor SA-57 from vehicle. Specifically, the majority (i.e., 23 of 24) of subjects learned to discriminate the dual FAAH-MAGL inhibitor SA-57 from vehicle within 40 training sessions. The 10 mg/kg SA-57 training dose was previously demonstrated to produce significant increases in brain levels of AEA and 2-AG (Wiebelhaus *et al.*, 2015). As SA-57 fully blocks FAAH activity at lower doses (0.05-1 mg/kg) than those required to inhibit MAGL (1.25-12.5 mg/kg) (Niphakis *et al.*, 2012) it provided a useful tool to examine the consequences of full FAAH inhibition while incrementally elevating brain 2-AG. The observation that 1 mg/kg SA-57, which produces maximal increases in endogenous AEA without detectable increases in 2-AG (Niphakis *et al.*, 2012), did not generalize to the training dose (10 mg/kg SA-57) indicates that FAAH inhibition alone is not sufficient to occasion to the SA-57 training dose. Similarly, neither FAAH inhibitor (i.e., PF-3845 or URB597) substituted for SA-57. In contrast, the dual FAAH-MAGL inhibitor JZL195, and two MAGL inhibitors, MJN110 and JZL184, fully substituted for the SA-57 training dose, suggesting that MAGL inhibition alone may be sufficient for generalization to the 10 mg/kg SA-57 training dose. Interestingly, PF-3845 produced an approximately two-fold leftward shift in the MJN110 substitution dose-response curve. The observation that rimonabant completely blocked the discriminative stimulus effects of SA-57 indicates that CB_1 receptors play a necessary role in the subjective effects of SA-57. Similarly, rimonabant completely blocked the substitution of both MAGL inhibitors (MJN110 and JZL184) and the dual FAAH-MAGL inhibitor JZL195. These findings suggest that elevating

endocannabinoid brain levels through the simultaneous blockade of FAAH and MAGL produces a CB_1 receptor mediated interoceptive stimulus.

Consistent with previous studies reporting that SA-57 or the dual FAAH-MAGL inhibitor JZL195 substitute for the THC discriminative stimulus (Long *et al.*, 2009; Hruba *et al.*, 2015; Walentiny *et al.*, 2015), we found that SA-57 (10 mg/kg) fully substituted for the discriminative stimulus effects of the potent cannabinoid receptor agonist CP 55,940 in C57BL/6J mice and the endogenous cannabinoid AEA (6 mg/kg) in FAAH (-/-) mice. The potency of SA-57 in producing a discriminative stimulus was similar to its potency in substituting for either CP 55,940 or AEA. Furthermore, SA-57's discriminative stimulus effects occurred at a training dose known to produce maximal increases in AEA and 2-AG (Niphakis *et al.*, 2012). In addition, CP 55,940 fully substituted for SA-57, an effect that was completely blocked by rimonabant, further implicating a pivotal role of CB_1 receptors in these effects. Similarly, the dual FAAH-MAGL inhibitor JZL195 dose-dependently substituted for SA-57. Time-course investigation revealed that SA-57 partially generalized at 0.5 h, fully generalized at 1 and 2 h, partially generalized at 4 h, and by 8 h mice responded mostly on the aperture paired with vehicle.

It is noteworthy that MJN110 and JZL184 fully substituted for the discriminative stimulus effects of SA-57, while mice treated with a low dose of SA-57 (which does not elevate 2-AG), URB597, or PF-3845 selected the vehicle aperture. These findings suggest that MAGL inhibition represents a driving force underlying the SA-57 training dose. However, the observation that PF-3845 increased the potency of MJN110 to substitute for SA-57 suggests that FAAH inhibition increases the effectiveness of the discriminative stimulus produced by MAGL

inhibition alone. The fact that 2-AG levels are approximately three orders of magnitude higher than AEA levels in wild type mouse brain (Ahn *et al.*, 2009; Long *et al.*, 2009) is consistent with the notion that MAGL inhibition elicits more prominent pharmacological effects than those produced by FAAH inhibition. Moreover, as FAAH is expressed on the post-synaptic terminal (Gulyas *et al.*, 2004), and MAGL (Dinh *et al.*, 2002) is expressed on the pre-synaptic terminal, it is plausible that AEA and 2-AG activate distinct CB_1 receptor mediated neuronal circuits.

Because AEA and 2-AG bind CB_1 and CB_2 receptors, AEA also binds TRPV1 receptors, and other FAAH substrates (i.e., PEA and OEA) bind PPARα receptors (Lo Verme *et al.*, 2005), we examined whether selective antagonists for each of these receptors would block the discriminative stimulus effects of SA-57. Rimonabant, but not the other receptor antagonists, completely blocked the discriminative stimulus effects of SA-57. These findings indicate that CB_1 receptor activation is required for the subjective effects of SA-57, while CB_2, TRPV1, and PPARα receptors are dispensable. Moreover, the fact that SA-57 did not affect ligand binding to CB_1 receptors in either a competitive or non-competitive manner is consistent with the hypothesis that it increases brain endocannabinoid levels that then elicit a CB_1 receptor-mediated discriminative stimulus.

The present study also assessed whether a variety of psychoactive non-cannabinoid drugs would substitute for SA-57. Specifically, nicotine did not substitute for the SA-57 training dose, although it significantly reduced response rates. In contrast, diazepam partially substituted for SA-57, but did so at a dose that reduced response rates. Similarly, diazepam partially substitutes for THC at high doses that produce motor impairment in the rat drug discrimination paradigm

(Wiley and Martin, 1999). Taken together, these studies suggest the possibility of a potential GABAergic component for CB_1 receptor-mediated discriminative stimuli. In addition, because MAGL inhibition reduces brain levels of arachidonic acid as well as various prostanoids (Nomura *et al.*, 2011), we tested whether the COX2 inhibitor valdecoxib would substitute for SA-57. However, valdecoxib was devoid of action in this assay, suggesting that prostaglandins do not play a necessary role in the discriminative effects of SA-57.

It is noteworthy that combined inhibition of FAAH and MAGL attenuates somatic signs of opioid withdrawal (Ramesh *et al.*, 2011). However, simultaneous blockade of these enzymes also elicits other cannabimimetic effects, as assessed in the tetrad assay including hypomotility, antinociception, catalepsy, and hypothermia (Long *et al.*, 2009; Anderson *et al.*, 2014; Ghosh *et al.*, 2015), as well as impaired performance in a Morris water maze spatial memory task (Wise *et al.*, 2012). These effects of dual FAAH and MAGL inhibition are similar to those of THC, whereas single inhibition of either enzyme produces a decreased spectrum and magnitude of cannabimimetic effects. Specifically, the MAGL inhibitor JZL184 produces antinociception, hypomotility, and dysregulation of thermoregulation when challenged with manipulations that elicit hypothermia (Nass *et al.*, 2015), while FAAH inhibition produces antinociception, but not other cannabimimetic effects (Long *et al.*, 2009). However, drug discrimination is more sensitive in detecting cannabimimetic effects compared with the tetrad assay. For example, THC is more potent in producing its discriminative stimulus effects than in eliciting the full set of tetrad effects (Long *et al.*, 2009; Marshell *et al.*, 2014a). Given that dual blockade of FAAH and MAGL significantly reduces locomotor activity (Long *et al.*, 2009) and SA-57 (10 mg/kg)

completely inhibits FAAH and MAGL activity (Niphakis *et al.*, 2012), the lack of rate suppressive effects of SA-57 (10 mg/kg) during training session is interesting. Similarly, THC (5.6 mg/kg) reduces locomotor activity, but does not reduce response rates during training sessions in drug discrimination procedure (Wiley *et al.*, 2005). This lack of apparent motor depression is consistent with the idea that rate suppressive effects of drugs undergo tolerance throughout the course of drug discrimination training (Solinas *et al.*, 2006).

The results of the present study suggest that SA-57 serves as a discriminative stimulus at doses that produce increased levels of both AEA and 2-AG through a CB_1 receptor mechanism of action, though elevated levels of 2-AG may be the main driving force for the SA-57 training dose. Although the brain regions mediating the discriminative stimulus effects of SA-57 and cannabinoid receptor agonists are unknown, it is noteworthy that endogenous cannabinoids, and their receptors are located in neural pathways mediating the reinforcing effects of drugs of abuse (i.e. mesolimbic dopamine pathway) (Oleson and Cheer, 2012).

In conclusion, the present study demonstrates that the dual FAAH-MAGL inhibitor SA-57 serves as a reliable discriminative stimulus. The observations that rimonabant completely blocks the SA-57 training dose, and mice trained to discriminate SA-57, CP 55,940, and AEA shows symmetrical substitution, strongly implicate the importance of the CB_1 receptor in this novel interoceptive stimulus. Collectively, these findings raise the provocative possibility that FAAH and MAGL serve as dual brakes to prevent the psychoactive consequences of CB_1 receptor over-stimulation caused by elevated levels of AEA and 2-AG.

Chapter 3. General Discussion

The first goal of this dissertation was to determine whether SA-57, a dual inhibitor of FAAH and MAGL, which regulates levels of the endocannabinoids AEA and 2-AG will serve as discriminative stimulus in the drug discrimination paradigm. To accomplish this goal, we established SA-57 as a discriminative stimulus, and then evaluated if each enzyme target of SA-57 (FAAH, MAGL, ABHD6) could substitute for the SA-57 discriminative stimulus. Until now, an inhibitor of endocannabinoid degradation has not been established as a discriminative stimulus. Then we determined the duration of action of the discriminative stimulus, and investigated which receptors were necessary for generating the discriminative stimulus. Next, we determined if inhibiting both FAAH and MAGL were required for generating the SA-57 discriminative stimulus, or if inhibiting either of these enzymes is sufficient. We discovered that inhibiting FAAH or ABHD6 alone had no effect, but MAGL inhibition alone substituted for SA-57 through a CB_1 receptor mechanism. Moreover, FAAH inhibition enhanced the potency of the MAGL inhibitor MJN110. In summary, the data from this dissertation indicate that FAAH and MAGL serve as endogenous breaks that prevent endocannabinoid overstimulation of CB_1 receptors. Also, the SA-57 discriminative stimulus can be used to examine potential subjective effects produced by other inhibitors of endocannabinoid hydrolysis.

5.1: Cross-substitution of SA-57 and CP 55,940

To select a training dose, we conducted a dose response study with SA-57 using two cannabinoids (AEA and CP 55,940) that are reported to serve discriminative stimuli to determine if SA-57 would substitute for either CP 55,940 (0.1 mg/kg) in C57BL/6J mice or AEA (5 mg/kg) in FAAH-/- mice. SA-57 (10 mg/kg) fully substituted for both the CP 55,940 and AEA discriminative stimulus, therefore, this dose of SA-57 (10 mg/kg) was selected to train a new cohort of mice. After mice learned to discriminate SA-57 (10 mg/kg), we observed that JZL195, and CP 55,940 fully substituted for SA-57. Because JZL195 is known to produce its behavioral effects through the CB_1 receptors (Long *et al.*, 2009), the substitution of JZL195 for SA-57, and cross-substitution between CP 55,940 and SA-57 provides strong evidence that CB_1 receptors are important for the SA-57 discriminative stimulus.

5.2: Investigating the receptors mediating the SA-57 discriminative stimulus

We evaluated whether the discriminative stimulus effects produce by SA-57 were mediated by CB_1, CB_2, PPARα or TRPV1 receptors. The CB_1 antagonist rimonabant (0.1 – 3 mg/kg) attenuated the SA-57 discriminative stimulus, but the lowest dose tested (0.03 mg/kg) was without consequence. The lowest dose of rimonabant (0.03 mg/kg) resulted in 70% of responses in the aperture paired with SA-57 indicating that as the dose of rimonabant decreased, more AEA or 2-AG was available to stimulate CB_1 receptors. It is interesting that lower doses of rimonbant (0.1 - 1 mg/kg) completely blocked the discriminative stimulus, because these doses have limited effects in other models of cannabinoid discrimination. For example, rimonabant (1

mg/kg) partially, but not fully attenuated the discriminative stimulus effects of rats trained to discrimination JWH-018 (0.3 mg/kg) (Wiley *et al.*, 2014). In rats trained to discriminate WIN 55,212-2, rimonabant (1 mg/kg) shifts the dose effect curve without attenuating the WIN 55,212-2 discriminative stimulus (Järbe *et al.*, 2011). Given the diversity of effects among studies investing the discriminative stimulus effects of cannabinoids, it is important to highlight one major experimental condition in the present dissertation. The sample size of mice in the rimonabant / SA-57 experiment (3-6 mice) was very small. This very small sample size may have contributed to the all-or-none effects observed by the different doses of rimonabant.

The CB_2 antagonists SR144528 had no effects on drug-like responding suggesting CB_2 receptors are not involved in this novel discriminative stimulus. Because SA-57 inhibits FAAH, and AEA binds PPARα and TRPV1 receptors in addition to degrading other N-acylethanolamines including N-palmitoylethanolamine (PEA), and N-oleoylethanolamine (OEA), we administered selective antagonist of PPARα and TRPV1 receptors to determine if either receptor contributed to the SA-57 discriminative stimulus. We selected antagonists of PPARα and TRPV1 receptors that were found to be effective at attenuating the effects of OEA and PEA in other behavioral assays (Lo Verme *et al.*, 2005; Kinsey *et al.*, 2009). We observed no change in the substitution pattern of SA-57 in the presence of either antagonist, suggesting PPARα and TRPV1 are not involved in the SA-57 discriminative stimulus. These findings along with the CB_1 antagonists study provide very strong evidence CB_1 receptors are the sole driver in the SA-57 discriminative stimulus. Also, CP 55,940 and MJN110 do not produce pharmacological effects through PPARα or TRPV1, and each drug fully substitutes for SA-57,

providing further evidence of the role of CB_1 receptors.

5.3: Investigating the eCB degradative enzymes mediating SA-57's effects

In the first part of this dissertation we established that mice could reliably learn to discriminate SA-57 from vehicle. Our next goal was to determine if inhibiting FAAH, MAGL or ABHD6 alone could substitute for SA-57, to determine the contribution of each enzyme in the discriminative stimulus effects of SA-57.

JZL195 is another potent inhibitor of both FAAH and MAGL. Because the high dose of JZL195 (20 mg/kg) fully blocks FAAH and MAGL activity, elevates AEA and 2-AG (Long *et al.*, 2009), and fully substituted for SA-57 (see figure 6) this supported the hypothesis that inhibiting both FAAH and MAGL were required to produce the SA-57 discriminative stimulus. However, until we examined selective inhibitors of each target of SA-57, it remained to be discovered which enzymes were required. Therefore, we employed selective inhibitors of FAAH, MAGL and ABHD6. The selective FAAH inhibitors (PF3845 and URB597) and the selective ABHD6 inhibitors (KT195 and KT182) did not substitute for SA-57, however the MAGL inhibitors (MJN110 and JZL184) fully substituted for SA-57, and was block by rimonabant. PF-3845 did enhance the potency of MJN110 inhibition, which suggests that FAAH inhibition can enhance the potency of MAGL inhibition. The observation that two MAGL inhibitors fully substitute for SA-57 provides strong evidence that maximal inhibition of MAGL is sufficient to produce subjective effects. Given that FAAH inhibition enhances the potency of MAGL inhibition, the subjective effects of SA-57 are probably attributed to inhibiting both enzymes.

The FAAH inhibitors PF-3845 and URB597 did not substitute for SA-57, and the mean responses for both inhibitors were below 5% for the aperture paired with SA-57. Finally, ABHD6 inhibitors did not substitute for SA-57. ABHD6 inhibition accounts for a small percentage of 2-AG degradation but is expressed post-synaptically so it regulates 2-AG in closer proximity to biosynthesis than MAGL, which is pre-synapatic.

Although FAAH inhibition does not substitute for SA-57, it produced a leftward shift in the MJN110 dose response, which is likely the result of an increase in AEA binding to CB_1 receptors. Because 2-AG levels are much higher than AEA levels in wild type mouse brain (Ahn *et al.*, 2009; Long *et al.*, 2009), it is more reasonable to anticipate that a MAGL inhibitor on its own would have a more robust effect than a FAAH inhibitor.

5.4: Nicotine and diazepam does not substitute for the SA-57 discriminative stimulus

As drug discrimination is considered a highly selective behavioral pharmacological assay to investigate the receptor mechanism of action of drugs, we employed agonists that do not stimulate CB_1 receptors such as nicotine and diazepam. As expected, nicotine and diazepam did not substitute for SA-57. However, diazepam did result in mean responses of 40% in the aperture paired with SA-57, indicating partial substitution for the SA-57 discriminative stimulus. This is similar to an older study observing partial substitution with GABA for THC (Mokler and Rosecrans, 1989). This partial effect could be because CB_1 receptors are localized on pre-synaptic GABA receptors. These observations indicate that drug discrimination is a useful preclinical model used to assess drug receptor activity *in vivo*.

5.5 Investigating the inhibition of arachidonic acid synthesis

Because MAGL is responsible for producing arachidonic acid and prostanoids in brain (Nomura *et al.*, 2011), we examined whether the COX-2 inhibitor valdecoxib, which reduces prostanoid synthesis but does not affect brain endocannabinoid levels, would substitute for SA-57. Valdecoxib produced minimal responses in the aperture paired with SA-57, suggesting that prostaglandins do not play a necessary role in the discriminative effects of SA-57.

5.5 Final Discussion

Previous studies have examined the ability of FAAH and MAGL inhibitors to substitute for the discriminative stimulus generated by direct CB_1 agonists (i.e. THC and CP 55,940). The purpose of the studies in this dissertation was to elucidate the effects of elevating endogenous cannabinoids, via inhibition of the degradative enzymes FAAH and MAGL, to serve as a discriminative stimulus. The present results support the hypothesis that elevating AEA and 2-AG levels by inhibiting their primary serine hydrolases FAAH and MAGL serves as a discriminative stimulus. The results presented here along with previous studies, indicate that endocannabinoid catabolic enzymes produce subjective effects that mimic the effects of THC. The discriminative stimulus effects of SA-57 were mediated via CB_1 receptors with no contribution by CB_2, TRPV1 or PPARα receptors.

We observed that complete blockade of MAGL substitutes for dual FAAH and MAGL inhibition, thus MAGL inhibition may produce subjective effects on its own. In this dissertation, the drug discrimination assay has provided valuable insights into the subjective effects produced

by inhibiting both FAAH and MAGL independently or in combination. Thus, SA-57 discrimination in mice is a very useful assay to investigate the subjective effects produced by inhibitors of endocannabinoid hydrolysis.

In summary, anandamide and 2-AG interact in a manner that is not fully understood to produce a CB_1 receptor mediated discriminative stimulus. Anandamide is reported to substitute for THC, but only when exogenous administration of AEA is combined in the presence of a FAAH inhibitor. Neither FAAH nor MAGL inhibitors produce THC-like subjective effects when administered on their own. However, increasing levels of endogenous anandamide and 2-AG mimics the effects of THC.

5.6: Future Studies

Given that MAGL inhibitors substitute for SA-57, it would be interesting to determine if MAGL inhibition alone can serve as a discriminative stimulus. This would provide an opportunity to directly investigate the subjective effects of MAGL inhibition as an alternative to administering MAGL inhibitors in mice trained to discriminate SA-57 or THC. To fully characterize the discriminative stimulus effects produced by the major endocannabinoid regulating enzymes, we would also need to determine if mice could discriminate a FAAH inhibitor. If a FAAH inhibitor serves as a discriminative stimulus, it would provide "direct evidence" that FAAH inhibition produces subjective effects. Given that FAAH inhibitors do not substitute for the discriminative stimulus effects of THC or SA-57 and do not produce the full subset of tetrad effects, we predict that a FAAH inhibitor would not serve as a discriminative

stimulus. Because MAGL inhibitors fully substitute for SA-57, it is likely that a MAGL inhibitor would serve as a discriminative stimulus. If complete blockade of MAGL serves as a discriminative stimulus, it would indicate MAGL inhibition produces subjective properties on its own, but these effects may not completely overlap with THC. Importantly, FAAH does not produce down regulation of CB_1 receptors. The lack of subjective properties without functional changes to the CB_1 receptor could indicate potential therapeutic benefits of FAAH inhibition without producing abuse-related subjective effects.

2-AG is reported to induce rapid increases in intracellular free Ca2+ concentrations in NG108-15 cells by stimulating CB_1 receptors (Sugiura *et al.*, 1999). Therefore, we know that 2-AG binds CB_1 receptors, however, we cannot measure changes in the binding of endogenous cannabinoids to CB_1 receptors after inhibiting endocannabinoid hydrolysis. In contrast, we can measure changes in the binding of CP 55,940 by determining the amount of ligand that displaces [^{3}H] CP 55,940, but we can't measure how much AEA or 2-AG displaces [^{3}H] CP 55,940 after administering SA-57. This capability would allow us to determine the amount of AEA and 2-AG that binds to CB_1 receptors after inhibiting endocannabinoid hydrolysis. For example, we would be able to determine the specific levels of AEA and 2-AG that is needed to bind CB_1 receptors to generate a discriminative stimulus, or substitute for a separate discriminative stimulus.

Also, it is yet to be determined which neural substrates are important for producing drug discriminative stimuli. Although, previous studies that conducted direct infusions of drug into specific brain regions have provided some insight into the brain regions that may be necessary for generating a discriminative stimulus. For example, nicotine infused directly into the medial

pre-frontal cortex (mPFC) fully substitutes for systemic injections of nicotine in the rat (Miyata et al., 1999). In a different study, two of six rats trained to discriminate i.p. injections of THC from vehicle selected the drug appropriate lever when THC was infused into the PFC or dorsal hippocampus (Mokler and Rosecrans, 1989). Also, infusions of vehicle into the reticular formation resulted in responses for THC indicating non-specific stimulation of this brain region can produce a THC-like discriminative stimulus. It is important to note that cannulae placements were not reported in this study. A similar approach could be taken in the future with mice trained to discriminate SA-57 using surgically implanted cannula aimed at the mPFC. Then we could infuse SA-57 into the mPFC to determine if the mPFC is necessary to generate discriminative stimulus produced by cannabinoids.

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